Leptin Induces Sca-1+ Progenitor Cell Migration Enhancing Neointimal Lesions in Vessel-Injury Mouse Models

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Abstract

Leptin is a hormone predominantly secreted by white adipose tissue which was initially believed to be solely a metabolic factor. Recent publications showed that leptin also played a role in inflammation and vascular disease, to which Sca-1$^+$ vascular progenitor cells within the vessel wall may contribute. However, the effect of leptin on vascular progenitor cells remains unknown.

In the present study, we sought to elucidate the effect of leptin on Sca-1$^+$ progenitor cells and neointimal formation, and to understand the underlying mechanisms.

Sca-1$^+$ progenitor cells from the vessel wall of C57BL/6J (Lepr$^{+/+}$) and db/db (Lepr$^{-/-}$) mice were cultured and purified with microbeads. The migration of Sca-1$^+$ progenitor cells *in vitro* derived from Lepr$^{+/+}$ mice was markedly induced by leptin. Western blotting and kinase assays revealed that leptin induced phosphorylation of STAT3, ERK1/2, FAK, and Rac1/Cdc42. Guide-wire injury of the femoral artery was performed in wild-type mice with neointimal development in a time-dependent manner. An increased expression of leptin in both injured vessels and serum was observed at 24 hours post-surgery. Red fluorescent protein (RFP) labelled-Sca-1$^+$ progenitor cells in Matrigel were applied to the adventitia of the injured femoral artery, RFP$^+$ cells were observed in the intima at 24 hours post-surgery, which significantly enhanced neointimal lesions at 2 weeks. This increase in neointimal lesions was reduced by pre-treatment of Sca-1$^+$ cells with CYT-354 (leptin antagonist). Moreover, guide-wire injury could only induce minor neointima in Lepr$^{-/-}$ mice at 2 and 4 weeks post-surgery. However, transplantation of Lepr$^{+/+}$ Sca-1$^+$ progenitor cells into the adventitial side of the injured femoral arteries of Lepr$^{-/-}$ mice significantly enhanced the neointimal formation.

In summary, upregulation of leptin levels in both the vessel wall and the circulation following vessel injury resulted in the migration of Sca-1$^+$

progenitor cells, which enhanced neointimal formation via leptin receptor-dependent STAT3- Rac1/Cdc42-ERK-FAK pathways.

Key words: Leptin, Sca-1^{+} progenitor, smooth muscle cells, neointimal formation, animal models

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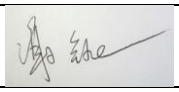
Candidate signature	
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Abbreviations

APC	Adventitial progenitor cells
α-SMA	Alpha-Smooth Muscle Actin
CCR	Chemokine (C-C motif) Receptor
CD	Clusters of Differentiation
CD11b	Integrin alpha M
CD29	Integrin beta-1
CD34	Hematopoietic progenitor cell antigen
CD45	Protein tyrosine phosphatase, receptor type, C
CD140b	Beta-type platelet-derived growth factor receptor
Cdc42	Cell division control protein 42 homolog
cDNA	Complementary DNA
c-kit	Proto-oncogene c-kit
CTR	Control
CXCR	Chemokine (C-X-C motif) Receptor
DAPI	4’,6-diamidino-2-phenylindole
Db/db	Leptin receptor deficient mice
ddH2O	Double distilled water
DMEM	Dulbecco’s Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
dNTPs	Deoxyribonucleotide triphosphates
ECs	Endothelial cells
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular-signal-regulated kinases
FACS	Fluorescence activating cell sorting
FAK	Focal Adhesion Kinase
FBS	Fetal bovine serum
Flk-1	Fetal Liver Kinase-1

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GTP	Guanosine Triphosphate
HE	Stain Haematoxylin and Eosin stain
HRP	Horseradish Peroxidase
IL	Interleukins
iPS cells	Induced Pluripotent Stem cells
JAK	Janus kinase
LEPR	Leptin receptor
LDL	Low Density Lipoprotein
LIF	Leukemia Inhibitory Factor
MEK	Mitogen-activated protein kinase kinase
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal Stem Cells
OB-R	Leptin receptor
PVAT	Perivascular adipose tissue
PDGF	Platelet-Derived Growth Factor
qPCR	Quantitative real time polymerase chain reaction
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog gene family, member A
RT	Reverse transcription
Sca-1	Stem cells antigen-1
SDS	Sodium Dodecyl Sulfate
SEM	Standard error of mean
SM22	Smooth muscle protein 22 alpha/transgelin
SMCs	Smooth muscle cells
STAT	Signal transducer and activator of transcription
ng	Nanogram
µg	Microgram
µL	Microlitre

Chapter 1
Introduction

1.1. Vascular System

1.1.1. The Definition of Blood Vessels

The blood vessels are the conduits which transport blood throughout the whole human body. There are five types of blood vessels: the arteries, which deliver the blood away from the heart; the arterioles, which extend and branch the arteries to capillaries; the capillaries, which carry out the exchange of oxygen, liquid and chemical compounds between the blood and tissues; the venules, which enable blood return from the capillaries to larger veins; the veins, which transport blood back to heart. The schematic representation of five types of blood vessels is listed below (Figure 1.1).

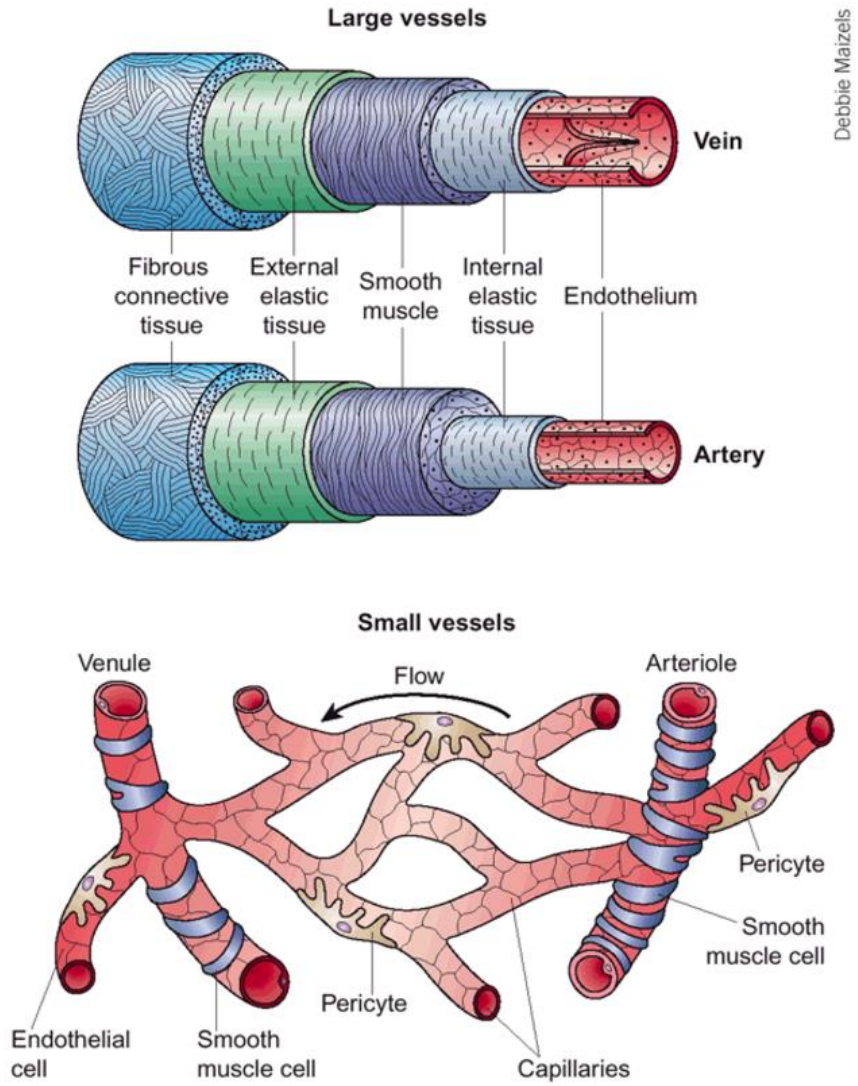


Figure 1.1. The structure of blood vessels. The scheme demonstrates the

structure of artery, arteriole, capillaries, venule and vein. The composition of vessel wall varies from different size of vessels. Large vessels such as arteries and veins have multiple layers of cellular and extracellular materials while capillaries only occupy a loose covering of pericytes (Cleaver & Melton, 2003).

1.1.2. The Structure of Vessels

Both arteries and veins have three layers in the vessel walls, which are named tunica intima, tunica media and tunica adventitia from the inside of the vessel walls outwards. Although arteries and veins have a similar composition of vessel walls, the middle layer, tunica media, is thicker in the arteries than it is in the veins, particular in large arteries.

The tunica intima, or intima for short, is the innermost layer of artery or vein, which is also the thinnest layer of the vessel wall. The intima consists of a single layer of endothelial cells (ECs) supported by an internal elastic lamina. Endothelium is a fine, transparent, colorless structure which is in direct contact with the blood flow. The endothelial layer can be easily observed when stained with PECAM-1 (CD31), VE-Cadherin (CD144) or silver nitrate. Unlike other four types of vessels, capillaries only have tunica intima, the ultra-thin structure of which enable the exchange of nutrients and gases in the capillary beds, suggesting the importance of the endothelium throughout the entire vascular system.

The tunica media, or media for short, is the middle layer of an artery or vein, which is between the tunica intima and tunica adventitia. Media is mostly composed of smooth muscle cells (SMCs) and elastic tissues. The media layer is much thicker in the large arteries than it is in the veins. The thicker media helps regulate the constriction and dilation of arteries, controlling the blood supply and blood pressure in the circulation. When SMCs in the media are stimulated by certain chemokines and factors, they contract and narrow

the vessel. This process is named vasoconstriction. On the contrary, vasodilation is the opposite term of vasoconstriction caused by the relaxation of the SMCs, decreasing blood flow and blood pressure. SMCs are usually identified by staining with alpha-smooth muscle actin (α-SMA), calponin, myocardin and smooth muscle myosin heavy chain (SMMHC),

The tunica adventitia or, adventitia for short, is the outermost layer of a vessel which is mainly composed of collagen and elastic fibers. The adventitia protects the vessels from overexpansion. The vessels are supported by external elastic lamina and collagen *via* anchoring the blood vessels to nearby organs and tissues. The adventitia of large blood vessels also contains nerves and nutrient capillaries (*vasa vasorum*) which provide the nutrition and oxygen to the adventitia. On the contrary, the media and intima are nourished by the diffusion of blood.

1.2. Atherosclerosis

1.2.1. The Definition of Atherosclerosis

Arteriosclerosis is the principal cause of heart attack, stroke, and gangrene of the extremities, remains a major contributor to the morbidity and mortality in the Western world (R. Ross & Glomset, 1976). Various factors, such as genetic polymorphism, hypercholesterolemia, modified lipoproteins, hypertension, diabetes mellitus, autoimmune responses, infections and smoking, were related to the development of cardiovascular diseases. However, the precise etiology and pathogenesis of these vascular diseases remains to be elucidated. Arteriosclerosis is characterized by the hyperplasia or hypertrophy of smooth muscle cells (SMCs) and accumulation of matrix protein in the intima and media with or without lipid deposition, both of which results in thickening and stiffness of the arterial wall (Stary, 1989). Arteriosclerosis is characterized into four subgroups: (spontaneous) atherosclerosis, accelerated arteriosclerosis (namely, transplant arteriosclerosis), restenosis after percutaneous transluminal coronary angioplasty and vein graft atherosclerosis (Wick, Kloflach, & Xu, 2004). The development of atherosclerosis is a very slow process, over a period of years even without any symptoms. In human, typical atherosclerosis undergoes three stages (Figure 1.2).

Early stage of atherosclerosis is associated with the adherence of circulating white blood cells (monocytes). Endothelial cells (ECs) are activated under certain circumstances such as hypertension, vascular injury and hypercholesterolemia. Meanwhile, the permeability of endothelium and the composition of extracellular matrix in the sub-endothelium layer have been altered, leading to the invasion and oxidation of low-density lipoprotein (LDL) particles from blood to endothelium. Initial lesions of endothelium result in the inflammatory response, which recruits the monocytes from the blood. Activated endothelial cells are capable of secreting various chemokines,

leading to the migration of additional leukocytes and the maturation of the monocytes coming from the circulation into macrophages. These macrophage as well as white blood cells infiltrate into the lesion, uptake the lipid and get trapped in the vessel wall to produce foam cells subsequently. Foam cells together with T lymphocytes constitute the fatty streak which is the earliest sign of atherosclerosis.

The second stage of atherosclerosis includes the migration of smooth muscle cells (SMCs) from media to the intima, prior to the proliferation of smooth muscle cells and secretion of extracellular matrix macromolecules such as collagen, elastin and proteoglycans. Smooth muscle cells and macrophages undergo apoptosis during the process of plaque formation, leading to the release of lipids in plaque's central zone. In typical atherosclerosis, three different areas can be identified by a different proportion of smooth muscle cells, macrophages, matrix and inflammatory cells: fibrous cap, lipid zone and basal zone. In a more advanced process, cholesterol crystal cores and microvessels are the biomarkers for atheromatous plaques. The final stage of atherosclerosis involves in some serious complications such as thrombosis, plaque rupture, calcification and intraplaque hemorrhage (Millon, Canet-Soulas, Boussel, Fayad, & Douek, 2014).

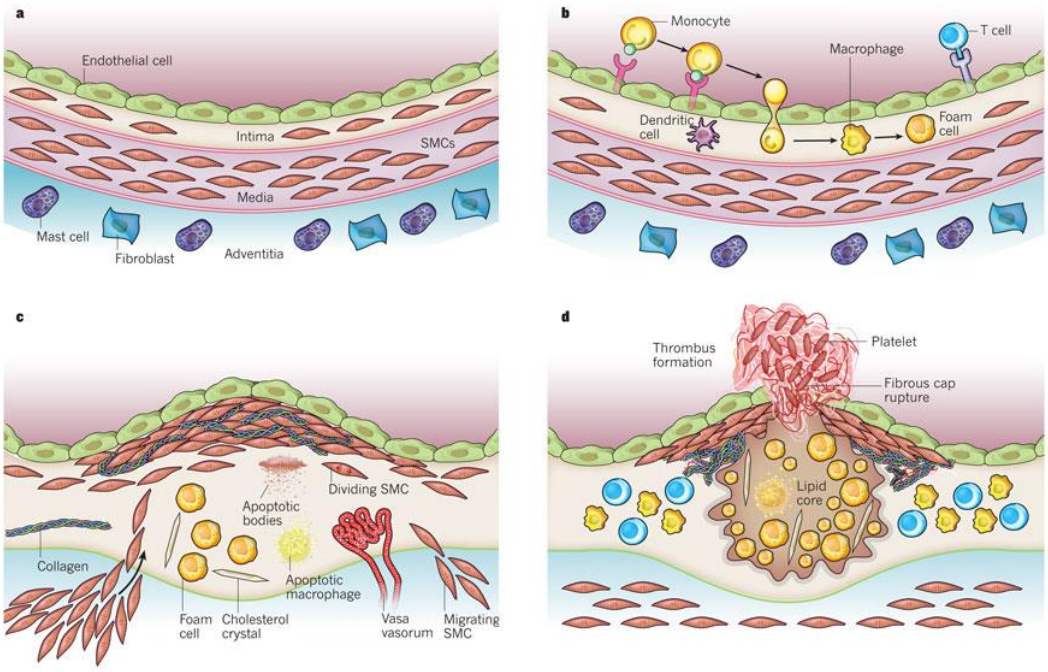


Figure 1.2. Stages of development of atherosclerosis (Libby, Ridker, & Hansson, 2011). a, A normal artery contains three layers. **b,** The initial stage of atherosclerosis is associated with adhesion of leukocytes to the activated ECs, migration of the attached leukocytes toward intima, and maturation of monocytes into macrophages which uptake lipid to produce foam cells. **c**, Lesion progression includes the proliferation of resident intima SMCs and media-derived SMCs. Plaque SMCs and macrophages can undergo apoptosis at the late stage of atherosclerosis, leading to a lipid or necrotic core. Cholesterol crystals and microvessels can be observed in an advanced plaque. **d**, The final stage of atherosclerosis comes to thrombosis, impeding entire blood flow.

1.3. Adult Cells in Atherosclerosis

1.3.1. Endothelial Cells Dysfunction

Vascular endothelial cells cover entire cardiovascular system from the heart to small capillaries. The endothelium is regarded as a semi-selective barrier and provides a non-thrombogenic surface. The dysfunction of endothelium is treated as a biomarker for various vascular diseases, which is often related to the early stage of atherosclerosis. The impaired endothelial function can be caused by hypertension, diabetes, hypercholesterolemia, etc. The endothelial lining can secret various autocoid factors which have an effect on other types of cells in vessel wall such as smooth muscle cells and pericytes, and in blood such as platelets and leukocytes. The endothelial isoform of NO synthase (eNOS) was one of the factors which were also shown to play important role in vascular remodeling (Palmer, Ashton, & Moncada, 1988). The basal level production of NO by endothelial cells is crucial for the homeostasis of the vascular system. Under pathologic situation, once NO is produced by eNOS, it can rapidly distribute through cell membranes as a paracrine factor. This secreted NO can have an effect on adjacent smooth muscle cells, circulating blood platelets and leukocytes by activating soluble guanylate cyclase, which catalyzes GTP to cGMP. NO may also be mediated by S-nitrosylation to alter the function of proteins reversely. The target protein of S-nitrosylation has been proved to regulate important cellular processes such as cell proliferation, apoptosis and ion channel activity (Gimbrone & García-Cardeña, 2016).

In the atherosclerotic region of the arteries, endothelium can be activated by various proinflammatory agonists such as interleukin-1 (IL-1), tumor necrosis factor (TNF), endotoxin, oxidized lipoprotein (ox-LDL) as well as biomechanical stimulation. The appearance of these agonists leads to a coordinated program of genetic regulation *via* nuclear factor-κB (NF-κB) within endothelial cells. Adhesion molecules such as vascular cell adhesion melocule-1 (VCAM-1) and secreted and membrane-associated chemokines

such as monocyte chemoattractant protein (MCP-1) can be upregulated subsequently, leading to the selective recruitment of monocytes and lymphocyte in the subendothelial area (Pober et al., 1986). The activated endothelial cells, smooth muscle cells, monocyte, macrophage and lymphocytes together result in the production of a complex mixture of paracrine cytokines and growth factors within the vessel wall, fostering the progression of atherosclerosis (Figure1.3).

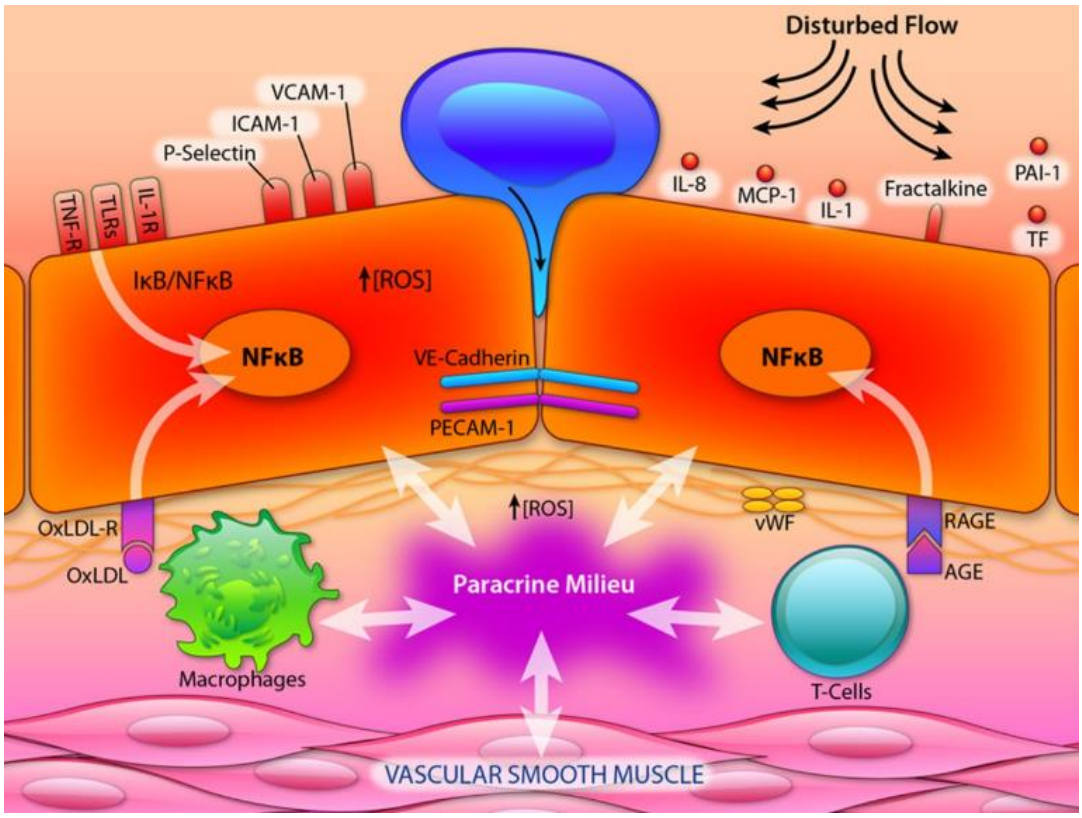


Figure 1.3. Endothelial proinflammatory activation (Gimbrone & García-Cardeña, 2016). In atherosclerotic regions, IL-1, TNF, ox-LDL and AGE secreted by leukocytes as well as MCP-1, IL-8, etc. from the disturbed blood flow activate the endothelium, followed by the activation of NF-κB in the nuclei, leading to a coordinated program of genetic regulation (VCAM-1, MCP-1, TF, vWF and PAI-1). This upregulation enhances the recruitment of leukocytes. The recruited leukocytes, together with activated endothelial cells, smooth muscle cell, and lymphocytes produce a complex paracrine milieu of

chemokines within the vessel wall, accelerating the process of atherosclerosis.

1.3.2. Leukocytes Recruitment

Following vascular injury and endothelial dysfunction, recruitment of leukocytes to the injured site is of great importance. Subsequent changes of adhesion events in endothelial cells ensure that the leukocytes leave the bloodstream and only stay at the damaged site (Ley, Laudanna, Cybulsky, & Nourshargh, 2007). Three groups of adhesion molecules can be defined between the attachment of leukocyte and endothelial cells: (1) Selectins are three carbohydrate-recognizing molecules with E-selectin expressing on the activated endothelium, P-selectin expressing on platelets and the endothelium, and L-selectin expressing on leukocytes (Vestweber, 1992); (2) Integrins are heterodimers with an α- and a β-chain which can recognize various ligands. Integrins need conformational changes to acquire fully functional adhesion ability. Leukocytes express integrins such as CD11 and CD18 (Hynes, 1992); (3) the immunoglobulin superfamily are the major ligands involved in leukocytes adhesion such as intercellular cell adhesion molecules (ICAM) 1-5, vascular adhesion molecule-1 (VCAM-1), platelet endothelial cell adhesion molecule-1 (PECAM-1) and junctional adhesion molecules (JAMs) which are expressed on endothelial cells (Langer & Chavakis, 2009).

The initial process of recruitment of leukocyte involves in the selectin-mediated interaction between leukocytes and endothelial cells. After the rolling or tethering of leukocytes on the luminal endothelial surface, leukocytes are capable of sensing the endothelial surface-bound chemokines, which are induced by proinflammatory factors (Charo & Ransohoff, 2006). Four groups of chemokines are defined according to the presence or absence of intervening amino acids between the first two N-terminal cysteine residues

which are CC, CXC, CX3C and C chemokines. Binding to the receptors of these chemokines on leukocytes leads to the activation of leukocytes' integrin, resulting in firm adhesion of leukocytes (Arfors et al., 1987). Besides the crosstalk between endothelium and leukocytes, platelet also plays a crucial role in the recruitment of leukocytes. Upon vascular injury, platelets aggregate in the inflammatory area and secret potent inflammatory and mitogenic substances which can significantly enhance the adhesion of endothelial cells and transmigration of leukocytes. A variety of chemokines such as stromal cell-derived factor 1 (SDF-1) can be released by platelets, which enable the activation of integrin of leukocytes and recruit more circulating leukocytes to the injured endothelium (Bleul, Fuhlbrigge, Casasnovas, Aiuti, & Springer, 1996). The interaction between platelets, endothelium and leukocytes mediated by P-selection on platelets and Mac-1 on leukocytes also significantly enhance the recruitment of leukocytes (Simon et al., 2000). In summary, the role of non-inflammatory cells such as platelets can also participate in leukocytes recruitment.

1.3.2.1. T Cell Infiltration

Recent studies showed that T lymphocytes could be detected both in early fibrous plaques and in more advance ones. The class II MHC antigen, HLA-DR which is inducible by T cell products are found expressing on smooth muscle cells in neointima, indicating that T lymphocytes may participate in atherogenesis (G. Hansson et al., 1988). Activated T cells are capable of releasing a series of angiogenic factors including vascular endothelial growth factor (VEGF) and stimulating early recruitment of leukocytes (G. K. Hansson, 2001). CD4 deficient mice showed an attenuated ability of angiogenesis in a hindlimb ischemia mouse model. What is more, inflammation in CD4 deficient mice was greatly impaired versus wild-type mice (Stabile et al., 2003). Immunohistochemistry staining of fatty streak of apoE$^{-/-}$ mice revealed that

$CD4^+$ T cells were prominent in the fibrous cap and subendothelial area, compared to $CD8^+$ T cells. CD25 subunit of IL2 which is a marker of activated T cells was also discovered in $CD4^+$-rich area. Taken together, activated $CD4^+$ T cells can participate in the formation of atherosclerosis (Xinghua Zhou, Stemme, & Hansson, 1996).

1.3.2.2. Monocyte / Macrophage Recruitment

Monocytes originate from the progenitor cells in bone marrow and migrate from the bloodstream to peripheral tissue. Upon inflammation or other pathologic situation, monocytes can give rise to macrophages and dendritic cells (Swirski et al., 2009). In the early stage of atherosclerosis, prominent monocytes were found attached to the endothelium of atherosclerotic area of the arteries (Figure 1.4), which is also a hallmark of atherosclerosis (Bobryshev, 2006).

According to the expression of chemokine receptor and specific surface markers, monocytes are divided into two main subsets. Monocytes which express high level of CC-chemokine receptor 2 (CCR2) but low levels of CX3C-chemokine receptor 1 (CX3CR1) are defined as inflammatory monocytes. Inflammatory monocytes occupy 2-5% of circulating leukocytes and can be rapidly recruited to the inflammatory areas (Serbina, Jia, Hohl, & Pamer, 2008). CC-chemokine ligand 2 (CCL2 or MCP1) and CCL7 (MCP3) are the ligands of CCR2 which mediates the recruitment of monocytes (Tsou et al., 2007). The expression of CCL2 in serum and tissues can be upregulated when proinflammatory cytokines or innate immune receptors are activated (Brown et al., 1994). CCL7 can be also induced during the infection in mice. Deletion of either CCL2 or CCL7 greatly decreased the recruitment of monocytes (Jia et al., 2008). However, the mechanism of how CCL2 and CCL7 work on recruitment remains unclear. CCL8 and 12 also bind to CCR2 but deletion of these genes does not affect the monocyte trafficking (Tsou et

al., 2007). Second major subsets of monocytes, also named LY6C^{low} express high levels of CX3CR1 and low levels of CCR2 and can adhere to and migrate along the luminal surface of endothelial cells (Auffray et al., 2007). CX3C-chemokine ligand 1 (CX3CL1) is the major ligand for this type of monocytes. Deletion of CX3CL1 abolished patrolling by LY6C^{low} monocytes and also diminished the recruitment of inflammatory monocytes (Auffray et al., 2009).

Monocytes migrate to arterial intima by penetrating through the endothelium. Once monocytes anchored to the endothelium of injured blood vessels, they undergo differentiation (Zernecke & Weber, 2005). During the differentiation of monocytes towards macrophages, CD68 is continuously expressed in the lysosomes of macrophages. M-CSF, which is the major regulator of differentiation of macrophages, was also detected in atherosclerotic lesions (Bobryshev, 2006). Furthermore, both macrophage populations exist in early atherosclerotic lesions at the same time. Most of macrophages with lipids in the cytoplasm are capable of transforming into foam cells, whereas the rest of the macrophages cannot become foam cells in the atherosclerotic area. Their functions, however, remain unknown. Besides the macrophages, monocytes can also differentiate into dendritic cells which are known as the most potent antigen-presenting cells (Bobryshev, 2005).

In more advanced atherosclerosis, only few macrophages and dendritic cells are detected in the fibrous caps (Bobryshev, Cherian, Inder, & Lord, 1999). The presence of dendritic cells can be observed mostly in the area of neovascularization, indicating the ingrowths of vasa vasorum from adventitia to intima (Bobryshev et al., 1999). In these neovascularization zones, E-selectin and ICAM-1 are highly expressed within the neovasculature. The expression of both factors mentioned above has been shown to be related to an increased accumulation of leukocytes in intima (Bobryshev & Lord, 1998).

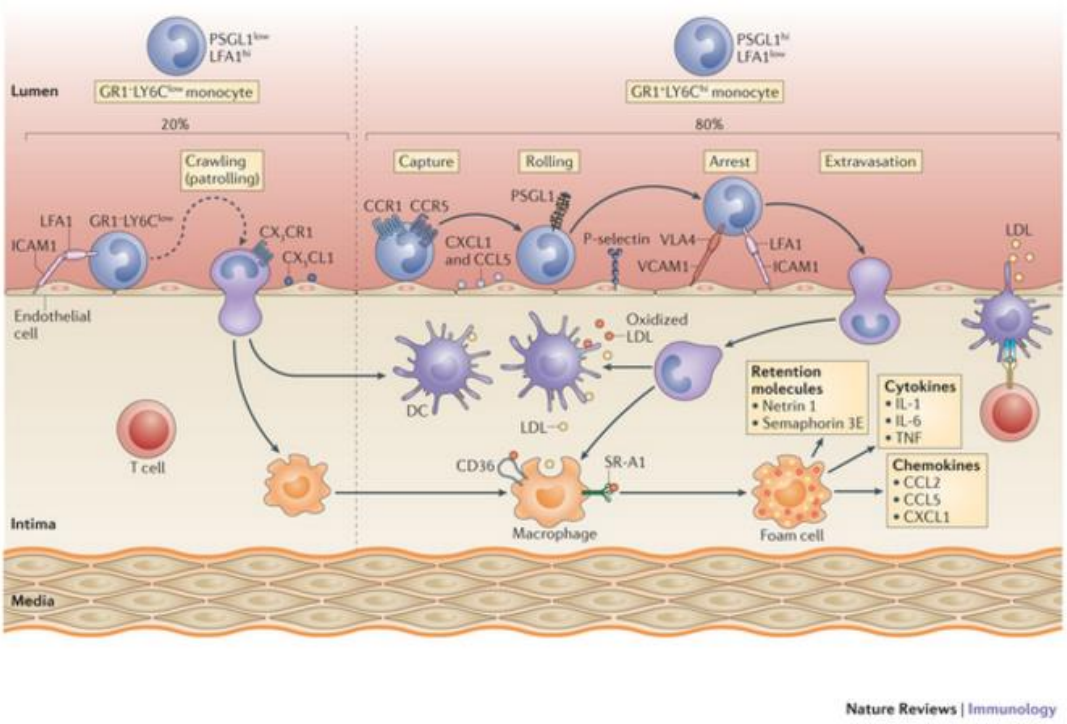


Figure 1.4. Recruitment of monocytes. Hyperlipidemia enhances the number of LY6C^{hi} monocytes and LY6C^{low} patrolling monocytes, both of which use various chemokine-chemokine receptor pairs for the infiltration to the intima. This process is promoted by endothelial adhesion molecules, such as ICAM1 and VCAM, followed by the differentiation into macrophages or dendritic cells (Moore, Sheedy, & Fisher, 2013).

1.3.3. Smooth Muscle Cells

Vascular smooth muscle cell (VSMC) is a particular type of smooth muscle which occupies the majority of the vessel wall. VSMC in normal arteries express contractile smooth muscle cell markers such as smooth muscle cell myosin heavy chain (MYH11), SM22α and smoothelin. Upon atherosclerosis, VSMC undergoes phenotypic switching to synthetic phenotypic, expressing markers such as S100A4, CX43, etc (Alexander & Owens, 2012). In some cases, VSMC can also occupy macrophage markers such as CD68. The phenotypic switching has been long-considered as an important factor in atherosclerosis, but its underlying mechanisms and biological effect remain

unclear. The origins of the majority of intimal smooth muscle cells within atherosclerotic lesions are commonly believed derived from medial smooth muscle cells which undergo phenotypic switching. These smooth muscle cells migrate into the intima, followed by the proliferation on site, secretion of extracellular matrix and participate in the formation of fibrous cap (Russell Ross, 1993). The underlying mechanism of phenotypic switching is complex. Myocardin$^{+/-}$ mice on apoE$^{-/-}$ backgroud showed an increased atherosclerosis with enhanced recruitment of macrophage compared to that in Myocardin$^{+/+}$ mice. Loss of myocardin greatly upregulated a series of pathways which were involved in inflammation and facilitated the phenotypic change from VSMC to a macrophage-like phenotype (Ackers-Johnson et al., 2015). Kruppel-like factor 4 (KLF4) has been shown necessary for phenotypic transition of VSMC *in vitro* in response to platelet-derived growth factor-BB (PDGF-BB). Knockout of KLF4 did not affect the total number of VSMC but significantly reduced the number of macrophage-like and mesenchymal stem cell-like cells, indicating the important role of KLF4 in smooth muscle cell transition (Shankman et al., 2015) (Figure 1.5). Meanwhile, several studies proposed alternative origins of VSMC-like cells in atherosclerotic lesions. For instance, Hu et al proposed that Sca-1^{+} progenitor cells may contribute to neointimal formation after vascular injury (Y. Hu et al., 2004). Adventitial pericytes (Tigges, Komatsu, & Stallcup, 2013) and adventitial fibroblasts (Coen, Gabbiani, & Bochaton-Piallat, 2011) may also be the potential sources for VSMC-like cells in atherosclerotic lesions.

VSMC is also responsible for producing extracellular matrix within the vessel walls during the initial stage of atherosclerosis, which in turn affects the lipid content and proliferative index in the plaque. Similar to macrophage, smooth muscle cells can also express various receptors for lipid uptake to form foam-like cells. Similar to endothelial, smooth muscle cells can express endothelial-like adhesion molecules such as vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, both of which can trap

monocytes and lymphocytes within the vessel wall. Smooth muscle cells can also secrete transforming growth factor-β, IFNγ, and MCP-1, all of which are proved to participate in atherosclerosis (Doran, Meller, & McNamara, 2008).

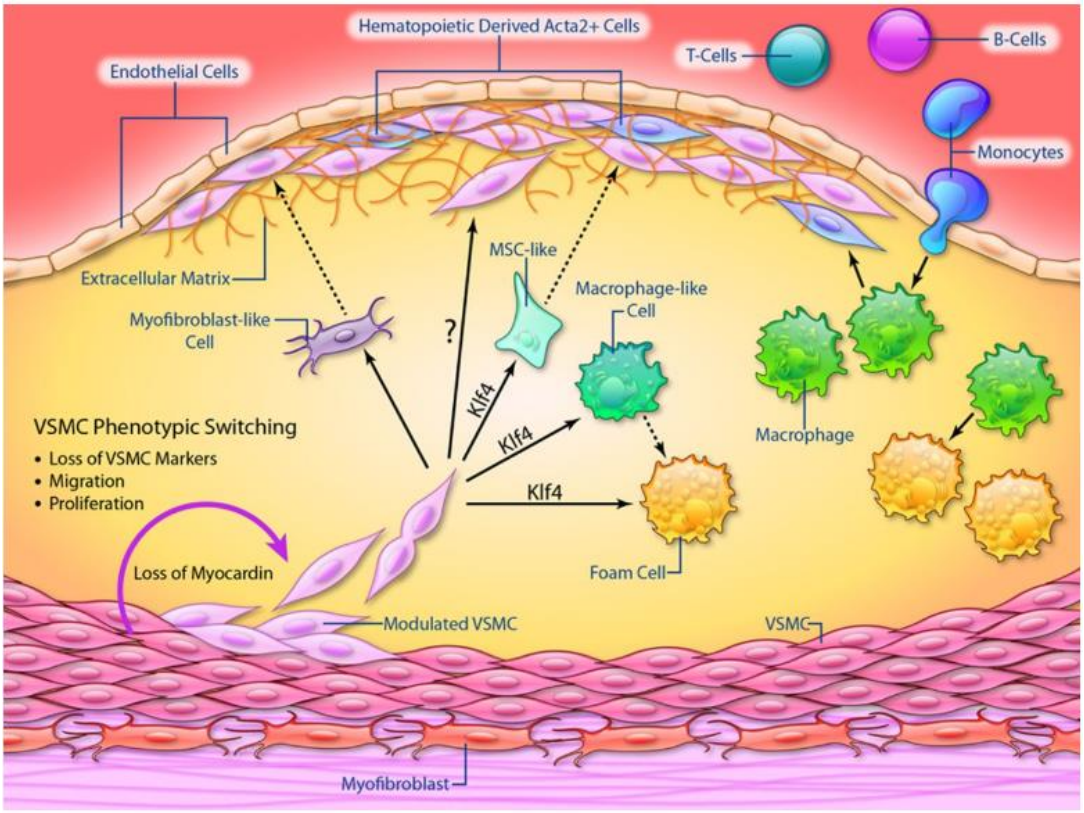


Figure 1.5. Schematic figure of the latest summary of the identification and origins of vascular smooth muscle cells in atherosclerotic lesions. The sold lines represent well-established pathways which contribute to atherosclerosis. Dotted lines or lines with "?" represent hypothesized pathways which were not fully understood (Bennett, Sinha, & Owens, 2016).

1.4. Stem Cells in Atherosclerosis

Stem cells are a group of undifferentiated cells which can differentiate into specialized cells. Stem cells are distinguished from other cells by two important features. First, self-renewal: stem cells are capable of producing more stem cells through mitosis and maintain their undifferentiated state. Second, potency: under certain conditions, stem cells can be induced into a specialized type of cells with particular functions (Basics, 2009) (Figure 1.6).

In mammals, there are two basic types of stem cells: adult stem cells and embryonic stem cells. Embryonic stem cells come from very early stage when a zygote begins to divide. Embryonic stem cells are able to differentiate into any kind of cells. On the other hand, adult stem cells have a limited multipotency and can only differentiate into limited type of cells (Basics, 2009). Vascular regeneration with the application of stem cells, which includes restoration of normal vascular structure and function, the reversal of vascular senescence and the growth of new blood vessels, may be a promising approach for the treatment of vascular diseases (Leeper, Hunter, & Cooke, 2010). Stem/progenitor cells are able to differentiate into vascular cell lineages, which may contribute to the regenerative process and could be useful for the disease treatment (Sun & Gerecht, 2009).

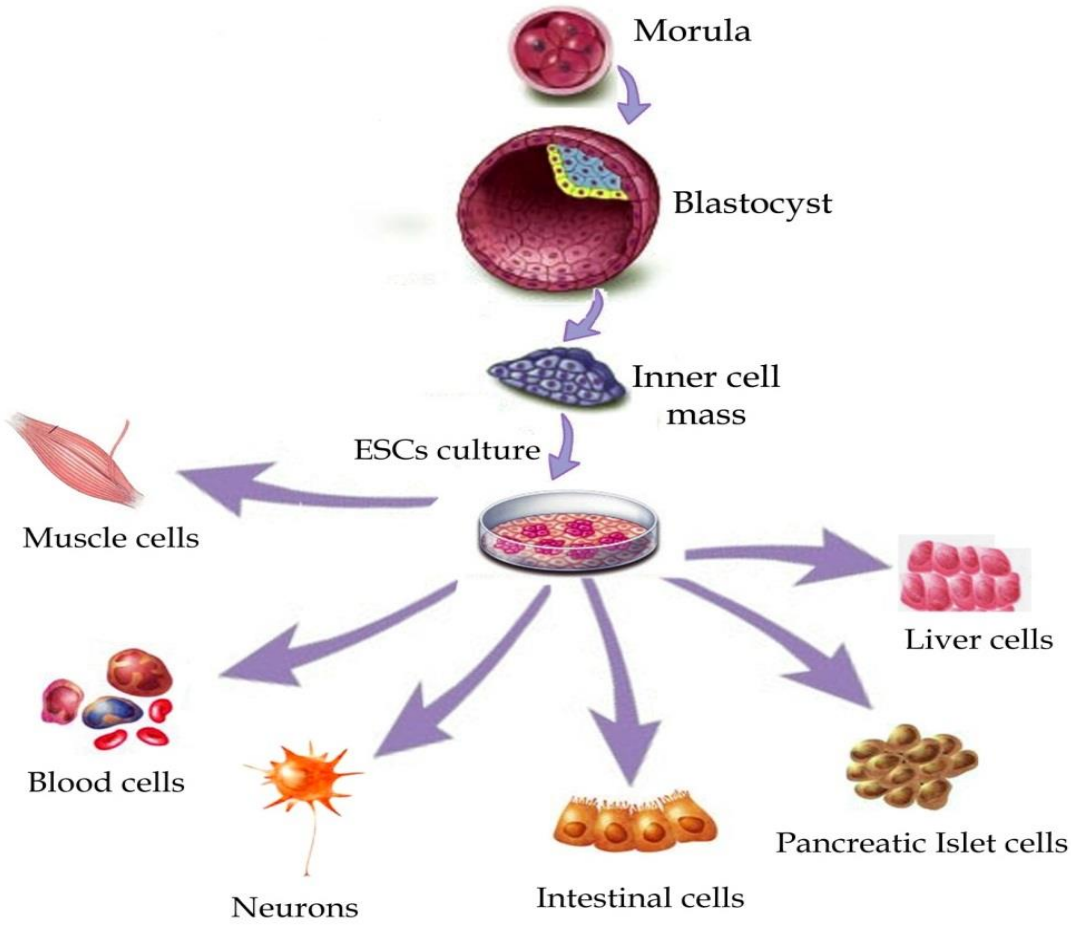


Figure 1.6. ESCs' differentiation. Human embryonic stem cells are capable of giving rise to different cell types including liver cells, intestinal cells, pancreatic islet cells, blood cells and muscle cells (Meregalli, Farini, & Torrente, 2011).

1.4.1. Embryonic Stem Cells

Embryonic stem cells (ESCs) are pluripotent cells, which are derived from the inner cell mass of the blastocyst. Embryonic stem cells have the ability to differentiate into any cell type and can divide indefinitely to produce daughter cells both *in vitro* and *in vivo* (Rippon & Bishop, 2004).

Various methods have been proposed for the differentiation of endothelial cells from embryonic stem cells. The mechanisms behind this have been studied in great detail (Luo, Wang, Wang, Xiao, & Xu, 2011). Bioengineered tissues are considered as a promising treatment in regenerative medicine. By

performing three-dimensional suspension culture and applying factors such as VEGF and 8-bromo-cAMP, stem cell-derived $CD31^+$ cells, were successfully generated of, and were able to form microvascular networks (Masuda et al., 2015). Co-culture of $CD31^+$ stem cell-derived cells with cardiomyocytes and dermal fibroblasts led to tube formation in cardiac cell sheets. This newly established method could be of great use for fabricating pre-vascularized cardiac cell sheet for grafting onto ischemic heart tissue or for preparation of a bioengineered heart on a scaffold (Masuda et al., 2015). To understand the mechanisms behind stem cell differentiation into endothelial cells, Wu et al. established a model to study epigenetic modification. They found that expression of histone demethylases KDM4A and KDM4C, which can bind to histones associated with Flk1 and VE-cadherin promoters, was upregulated during endothelial differentiation. Deletion of either KDM4A or KDM4C inhibited endothelial differentiation in murine embryonic stem cells *in vitro* and retarded blood vessel formation in zebrafish *in vivo* (Wu et al., 2015).

Regarding differentiation of embryonic stem cells towards smooth muscle cell, there are a large number of reports about the mechanisms behind cell differentiation (Q. Xiao, Wang, Luo, & Xu, 2010). Recently, Wang et al (X. Wang, Karamariti, Simpson, Wang, & Xu, 2015) reported that the expression of dickkopf homolog 3 (DKK3) was essential for the expression of smooth muscle markers and myocardin at both the mRNA and protein levels during the differentiation of stem cell towards smooth muscle cells. Overexpression of DKK3 led to a subsequent upregulation of the markers mentioned previously. Further investigation indicated that DKK3 induced the activation of activating transcription factor 6 (ATF6), leading to the firm binding of ATF6 to the myocardin promoter and increasing its expression (X. Wang et al., 2015). These findings offered a novel mechanism by which DKK3 regulated stem cells' differentiation by activating ATF6 and promoting myocardin expression.

1.4.2.Induced Pluripotent Stem Cells

Unlike embryonic stem cells, induced pluripotent stem (iPS) cells are pluripotent stem cells which can be directly generated from adult cells. This technology was firstly developed by the Yamanaka's group in 2006 *via* the application of four specific gene encoding transcription factors, Oct4, Sox2, cMyc and Klf4 in adult fibroblasts, which can be used to convert mature cells into pluripotent stem cells (Takahashi & Yamanaka, 2006) (Figure 1.7).

Some studies then focused on the differentiation of iPS cells towards vascular lineages. Human iPS cells derived from patients with type 1 diabetes mellitus display similar differentiation efficiency when compared with iPS cells derived from healthy donors (Chan et al., 2015). These patient-derived iPS cells can differentiate into endothelial lineages expressing typical endothelial markers and with functional capabilities of lectin binding, acetylated low density lipoprotein uptake, tube formation in Matrigel and response to TNF-α. IPS-derived endothelial cells could undergo morphogenesis and assemble into 3D networks when cultured in engineered hyaluronic acid hydrogels or in response to low oxygen *in vivo*, and could incorporate into developing vasculature in zebrafish (Chan et al., 2015). Similar results were also obtained in another study where human iPS cells derived from somatic tissue could differentiate into both endothelial cells and pericytes which were fully functional both *in vivo* and *in vitro* (Orlova et al., 2014).

To further investigate the mechanisms of iPS cell differentiation, Chen et al discovered that miR-199b was involved in endothelial differentiation (T. Chen et al., 2015). A step-wise increase in expression of miR-199 was detected during endothelial differentiation. Notably, miR-199b targeted the Notch ligand JAG1, resulting in VEGF transcriptional activation and secretion through the STAT3 transcription factor. Upon shRNA-mediated knockdown of the Notch ligand JAG1, the regulatory effect of miR-199b was ablated, and there was robust induction of STAT3 and VEGF during endothelial differentiation (T.

Chen et al., 2015). Using an *in vitro* tube formation assay and implanted Matrigel plugs *in vivo*, miR-199b was also shown to regulate VEGF expression and angiogenesis. MiR-199b can thus be said to play a novel role as a regulator of the phenotypic switch during vascular cell differentiation from iPS cells by regulating critical angiogenic signaling responses.

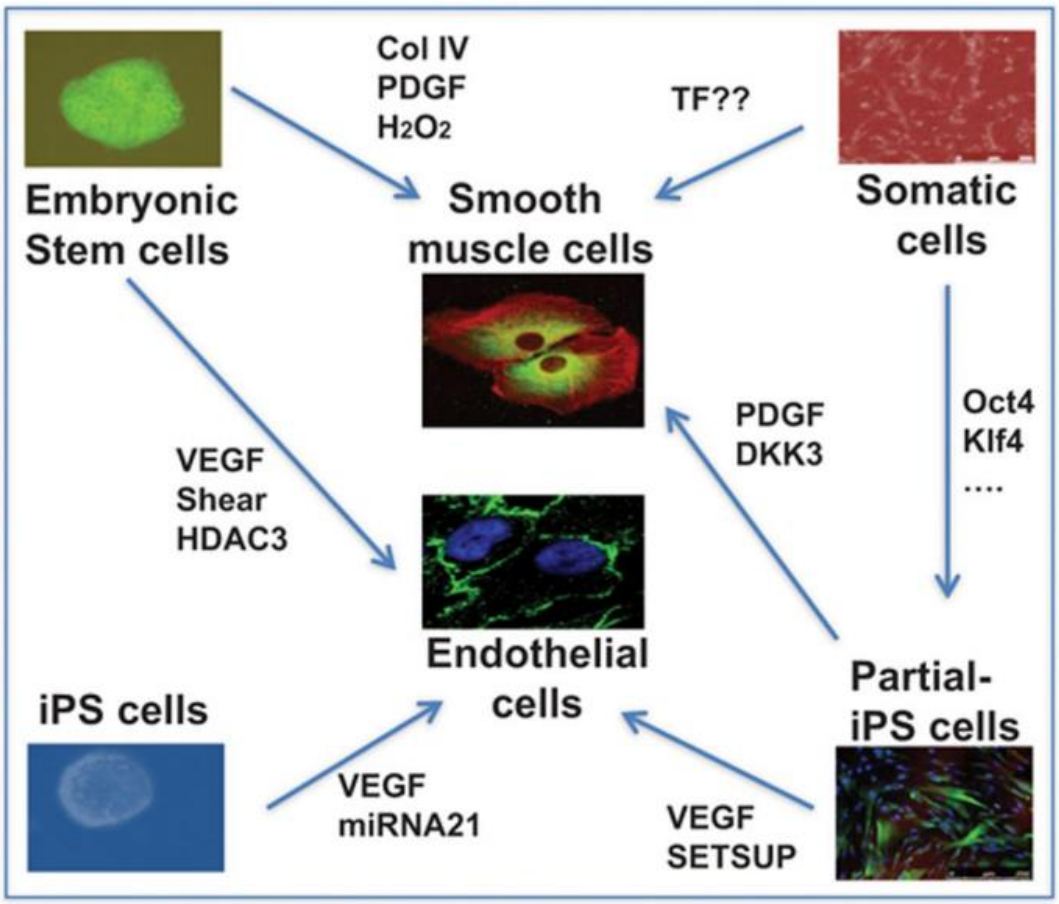


Figure 1.7. Embryonic stem cells and induced pluripotent stem cells can give rise to vascular lineages. ESCs can differentiate into endothelial cells and smooth muscle cells *via* multiple signaling pathways (Li Zhang & Qingbo Xu, 2014). For somatic cells, they can be reprogrammed and re-differentiated into vascular lineages.

1.4.3.Adult Stem Cells

Adult stem cells are undifferentiated cells found among the differentiated cells in tissue or organs. They are also known as non-embryonic stem cells or

somatic stem cells, which have the ability to divide indefinitely and differentiate into all cell types of the organ where they locate (Toma et al., 2001). Several signaling pathways are involved maintaining their abilities of self-renewal and potency, such as Notch, Wnt and TGFβ (Beachy, Karhadkar, & Berman, 2004; Dontu et al., 2004). Adult stem cells can be isolated from many regions including bone, bone marrows, blood and adipose tissues. Other adult tissue sources include but not limited to nose, muscle, liver, skin, brain, and the retina of eye (Kumar, 2008).

1.4.3.1. Mesenchymal Stem Cells

Mesenchymal stem cells are a group of multipotent stromal cells which can differentiate into various types of cells, such as osteoblasts, chondrocytes, myocytes and adipocytes (Abedin, Tintut, & Demer, 2004) (Figure 1.8). Mesenchymal stem cells can be isolated from a number of different tissues including umbilical cord, bone marrow, and adipose tissue (Ding, Shyu, & Lin, 2011). The properties of these cells vary from tissue to tissue. For instance, cells isolated from adult bone marrow display a stable phenotype, including SH2, SH3, CD29, CD44, CD71, CD90, CD105, CD106, CD120a, CD124 expression and are capable of differentiating into adipocytic, chondrocytic, or osteocytic lineages (Pittenger et al., 1999). Understanding the mechanisms behind cell differentiation, migration, mobilization and homing will be of great help in the future clinical application when these cells are regarded as treatments for vascular diseases.

Due to their easy access and relative abundance compared to other kinds of mesenchymal stem cells, adipose tissue stromal cells have recently become a popular cell type for stem cell research (Preda et al., 2014). Adipose tissue is a highly complex mixture which consists of more than 90% tissue volume adipocytes and stromal vascular fraction (SVF) (Weisberg et al., 2003). SVF is a heterogeneous cell population with adipose derived stem

cells (ADSCs) inside. ADSCs isolated from human subcutaneous adipose tissue displayed specific antigenic phenotype: $CD34^+$, $CD13^+$, $CD45^-$, $CD14^-$, $CD144^-$ and $CD31^-$ with the capacity of giving rise to adipocytes or endothelial cells *in vitro* (Planat-Benard et al., 2004). Specific ADSCs which expressed Sca-1^+, $CD34^+$, $CD45^-$ and $CD31^+$ were capable of differentiating into white adipose tissue in mice (W. Tang et al., 2008). One article also demonstrated that the proliferative ability of $CD34^+$ ADSCs could be maintained for 20 weeks *in vitro* (Yoshimura et al., 2006), whereas $CD34^-$ ADSCs were more plastic but less proliferative (M Bailey, Kapur, & J Katz, 2010).

The role of ADSCs in vascular remodeling was heavily studied. Injection of allogeneic abdominal adipose-derived stem cells, either with or without transfection with plasmid-VEGF165, provided a protective role in a rabbit model of critical hindlimb ischemia. These adipose-derived stem cells increased arteriolar density and protection against ischemia-induced muscle lesions (Olea et al., 2015). Although cell culture provides a good approach for studying the differentiation, migration, and proliferation of ADSCs *in vitro*, it is more relevant to study ADSCs in their biological context *in vivo*. SVF is obtained by collagenase digestion of adipose tissue and centrifugation to remove mature adipocytes. Joe et al. purified Lin^-Sca-1^+CD34^+ cells from SVF from white adipose tissue in GFP mice by FACS (Joe, Yi, Even, Vogl, & Rossi, 2009). The cells were then transplanted with Matrigel into wild-type mice. As a result, adipocyte progenitor cells were able to form mature unilocular and multilocular adipocytes with GFP expression. In another research, Miranvile et al. reported that $CD31^-CD34^+$ SVF cells could differentiate into endothelial cells, express CD31 markers and develop vascular structure in Matrigel in response to the treatment with VEGF, suggesting adipose derived stem cells may participate in an angiogenic process (Miranville et al., 2004).

Though mesenchymal stem cells have a therapeutic benefit, recent studies suggested they may also play a role in the pathology of aortic valve disease

(Wirrig & Yutzey, 2014). One potential mechanism is about the regulation of endothelial-to-mesenchymal transitions (EMT) during stem cell differentiation. The endocardial endothelial cells undergo EMT, producing mesenchymal valve progenitor cells in hyaluronan-rich extracellular matrix. Sox9 is required for the proliferation of endocardial cushion and the expansion of valve progenitor cells. Deletion of Sox9, which is a valve progenitor marker, resulted in the calcification of aortic valve in the mouse model, illustrating a potential role of these progenitor cells in heart valve diseases, the mechanism of which could be useful in the future treatments (Wirrig & Yutzey, 2014). Besides adipose tissue, bone marrow, heart valve mentioned above, it seems likely that further sources of mesenchymal stem cells exist.

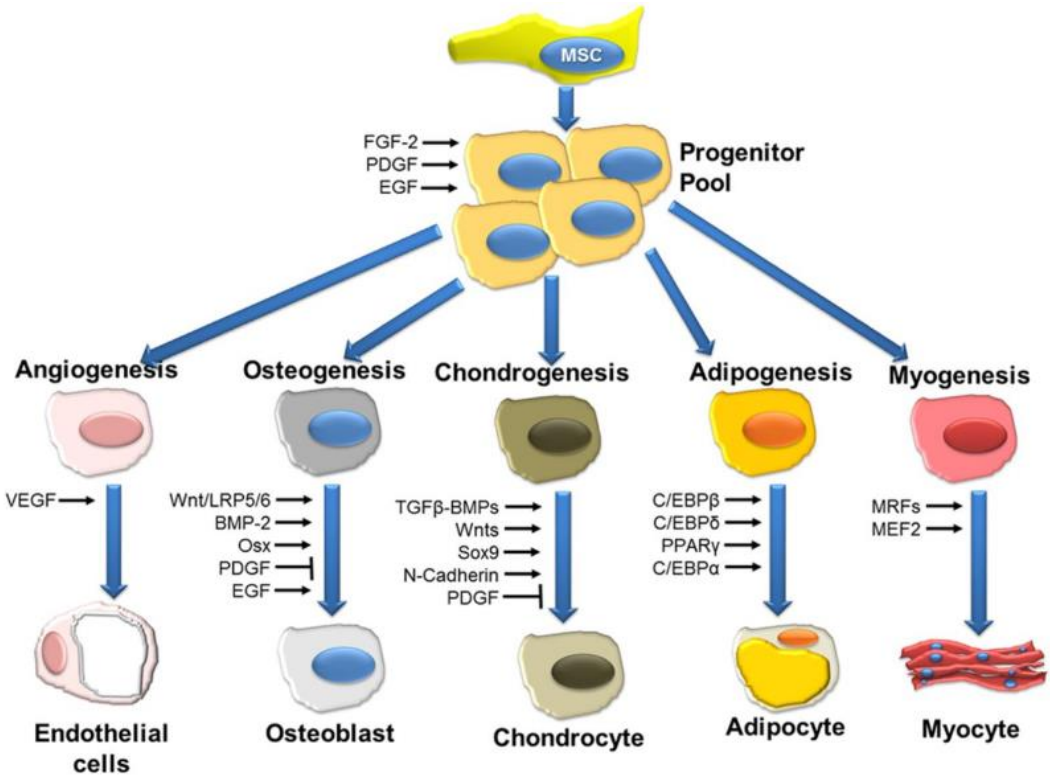


Figure 1.8. MSC differentiation. Mesenchymal stem cells can be isolated from various tissues, such as umbilical cord, bone marrow, cartilage, muscle, and adipose tissue. Cells from different tissues differ in properties including cell surface markers expressed and differentiation abilities. Mesenchymal stem cells can give rise to many cell lineages, such as osteocytes,

chondrocytes, and fibroblasts. Under certain circumstances, mesenchymal stem cells in the circulation can migrate to an injured area, differentiating into endothelial cells to repair damage-triggering angiogenesis and smooth muscle cells (SMC) which potentially form neointimal lesions. Schematic summary of involved molecules and transcription factors during the differentiation of mesenchymal stem cells are shown above (Karantalis & Hare, 2015).

1.4.3.2. Hematopoietic Stem Cells

Hematopoietic stem cells are a group of stem/progenitor cells which can give rise to both myeloid and lymphoid lineages of blood cells. They are found in the largest quantities in bone marrow but also exist in peripheral and umbilical cord blood. The physiological and pathological state of hematopoietic stem cells is vital for vascular regeneration (Mathur & Martin, 2004). $ApoE^{-/-}$ mice with monocytosis and atherosclerosis showed enhanced expansion of these cells associated with increased surface expression of a common β subunit of granulocyte macrophage colony-stimulating factor/interleukin-3 receptor (M. Wang et al., 2014). Transplantation of $apoE^{-/-}$ bone marrow into $LDLR^{-/-}$ mice resulted in an increased level of granulocyte macrophage colony-stimulating factor on stem cells and innate response activator B cells in spleen, which led to the expansion of hematopoietic stem cells. Therefore, granulocyte macrophage colony-stimulating factor could contribute to monocytosis and increased damaged macrophage content, affecting atherosclerosis plaque stabilization (M. Wang et al., 2014). Scavenger receptor type B1, as a high density lipoprotein receptor, was found on murine hematopoietic stem cells. The expression of SR-BI was related to the anti-proliferative effects of HDL on hematopoietic stem cells. $SR\text{-}BI^{-/-}$ bone marrow transplanted $LDLR^{-/-}$ mice induced reconstruction of white blood cells, production of inflammatory cells and plaque development. Moreover, HDL level was negatively correlated with

the proliferation and differentiation of hematopoietic stem cells in the circulation, which was associated with atherosclerosis progression (M. Gao et al., 2014). Another type of bone marrow-derived cells, megakaryocytes, is responsible for platelet production. Recently a group of scientists confirmed that the ATP-binding cassette transporter B6 was highly expressed in megakaryocyte progenitors (Murphy et al., 2014). Deletion of the ATP-binding cassette transporter B6 resulted in the expansion of megakaryocyte progenitors, increased platelet counts and platelet activity. Abcb6$^{-/-}$ bone marrow–transplanted mice expressed higher levels of C-C motif ligand 5 and accelerated atherosclerosis, leading to increased macrophage accumulation in atherosclerotic plaques (Murphy et al., 2014). Taken together, hematopoietic stem cells exert their effectiveness on atherogenesis *via* differentiation into macrophages/platelets or influencing lipid metabolisms.

1.4.3.3. Direct Reprogramming of Vascular Cells

Transplantation of endothelial cells is a promising treatment for the repair of damaged blood vessels and the growth of new ones after ischemia. However, in development of strategies based on the differentiation of pluripotent stem cells researchers have found these cells to have limited proliferative potential and instable function, presenting a problem when trying to generate enough functional endothelial cells for therapy. To address this problem new reprogramming protocols have recently been developed (Morita et al., 2015). The transcription factor Ets variant 2 (Etv2) is known to be essential for the specification of endothelial and hematopoietic lineages in early gestation. Etv2 was found capable of directly converting primary human adult skin fibroblasts into functional endothelial cells in combination with FOXC2 through a composite DNA-binding site (Morita et al., 2015). In a more complex protocol Etv2 was also shown to be able to directly reprogramme human amniotic cells into endothelial cells without an intermediate pluripotent

state. In this protocol Etv2 is expressed for two weeks, followed by the expression of transcription factors FLI1 and ERG1, while TGF-βis inhibited for 3 weeks. Using this method, human mid-gestation lineage-committed amniotic fluid–derived cells were converted into a stable, functional and proliferative population of vascular endothelial cells. Both of these protocols involving Etv2 could be a promising in producing a large pool of endothelial cells for treatment (Ginsberg, Schachterle, Shido, & Rafii, 2015).

Direct reprogramming of adult cells can also be accomplished with the application of viral vectors encoding other transcription factors. In one study overexpression of eleven candidate genes which are key regulators of endothelial development was achieved by lentiviral infection of adult skin fibroblasts from a Tie-GFP mouse, leading to an efficient reprogramming of skin fibroblasts into endothelial cells (Han et al., 2014). The function of derived endothelial cells was confirmed both *in vitro* and *in vivo* (Han et al., 2014). Sayed et al have recently pointed out that viral vectors are more than just passive vehicles for transcription factors due to their participation in the process of nuclear reprogramming to pluripotency. They manage to transdifferentiate human fibroblasts into endothelial cells by the application of viral vector carrying the ubiquitin promoter driving firefly luciferase (Fluc). These induced endothelial cells were also verified by a series of functional assays both *in vivo* and *in vitro* (Sayed et al., 2015).

1.4.3.4. Vascular Progenitor Cells

The presence of vascular resident stem cells has been determined in all three layers of the blood vessel wall, the intima, media and adventitia (L. Zhang & Q. Xu, 2014) (Figure 1.9). Investigation on murine vessels revealed that Sca-1$^+$ progenitor cells exist in the adventitia of large and medium sized vessels, and are able to migrate into the intima when seeded on the adventitial surface in Matrigel (Y. Hu et al., 2004). This Sca-1$^+$ progenitor cells derived from

adventitia of aortic artery are prone to differentiate into smooth muscle cells *in vitro*. Besides adventitia, sca-1^{+}/Lin^{-}/c-kit^{-}/CD34^{-} cells were found in the media of mouse thoracic/abdominal aorta and were shown to differentiate into endothelial cells or smooth muscle cells in response to VEGF or PDGF in culture medium (Sainz et al., 2006). In addition, the intima was also proven to have endothelial progenitor cells which could contribute to vascular regeneration (Covas et al., 2005; Ingram et al., 2005). The ratio between endothelial and smooth muscle cells remains stable under normal physiological conditions but is altered markedly in the pathogenesis of vascular diseases. Accumulating evidence demonstrates that vascular stem/progenitor cells may contribute to this process and lead to vascular regeneration (Evelyn Torsney & Qingbo Xu, 2011). Furthermore, it was reported there are resident macrophage progenitor cells in the adventitia of the mouse aorta. These Sca-1^{+}/CD45^{+} progenitor cells were not derived from circulation and were found at higher levels in a mouse model of atherosclerosis, which provides further evidence that vascular resident progenitor cells are a heterogeneous population (Peter J Psaltis et al., 2014). More information about the vascular progenitor cells is listed in table 1.1.

Vascular resident stem/progenitor cells have a high potential in cardiovascular regeneration therapies (E. Torsney & Q. Xu, 2011). In a recent study, adventitial progenitor cells were harvested from veins during coronary artery bypass graft (Iacobazzi et al., 2014). These progenitors showed antioxidant properties with up-regulation of antioxidant enzymes such as superoxide dismutase and catalase, the effects of which can be abolished by pharmacological inhibition of superoxide dismutase. In a mouse limb ischemia model, injection of these progenitor cells induced neovascularization which offered protection from ischemia (Iacobazzi et al., 2014). The role of human saphenous vein-derived adventitial progenitor cells was investigated in a similar mouse model of limb ischemia. Transplanted cells reached their therapeutic target and triggered revascularization of

ischemic limbs. In addition, FLT-1 gene silencing in these progenitor cells markedly reduced their ability to form tubes, confirming the outcomes from predicted expressional studies (Gubernator et al., 2015). The differentiation of vascular progenitor cells plays crucial roles in vascular diseases (Figure 1.10). Therefore, medications which are involved in cell differentiation were heavily investigated. Resveratrol is a natural phytochemical which was recently found to induce vascular stem/progenitor differentiation into endothelial cells, which express CD31 and eNOS with the great reduction of miR-21, Akt phosphorylation and nuclear β-catenin expression. Progenitor cells applied with resveratrol treatment demonstrated an enhanced ability for re-endothelialization *ex vivo* and reduced lesion formation was seen with a resveratrol diet in a mouse model of vessel graft (Campagnolo et al., 2015).

Vascular disorder in the lung could be a lethal disease at the final stage, and is related to the loss of functional microvasculature (Frid et al., 2006). Progenitor cells had been found in lungs adjacent to small vessels (Nadaud et al., 2015). These cells can differentiate into smooth muscle cells *in vitro*. *In vivo* an increased recruitment of these progenitors in response to chronic hypoxia was observed. Subsequently, these progenitors can differentiate into smooth muscle cells in the remodeled vessels, suggesting the participation of the progenitor cells in vascular remodeling (Nadaud et al., 2015). Furthermore, resident endothelial cells and progenitors were capable of proliferation and restoring endothelial barrier function after inflammatory lung injury (Mao, Ye, Liu, Song, & Liu, 2015). The suppression of endothelial and progenitor cell proliferation by blocking intrinsic nuclear factor-κB at the barrier repair phase was associated with an augmented endothelial permeability and impeded endothelial barrier recovery (Mao et al., 2015). In lungs, 8 weeks after injury, both endothelial and progenitor cells contribute to endothelial repair for endothelial barrier restoration.

Sca-1$^+$ markers did not express in human beings. Thus more markers representing vascular progenitor cells were involved. Gli1$^+$ perivascular cells

expressed typical MSC markers, exhibit trilineage differentiation *in vitro* and substantially contribute to organ fibrosis *in vivo* (Kramann et al., 2015). Experiments in large animal models are of great value where possible. One group managed to isolate a population of $CD44^{+}CD56^{+}CD90^{+}CD105^{+}CD34^{-}CD45^{-}$ progenitor cells from the porcine aorta, which was shown to be useful for vascular regeneration in the pig model (Zaniboni et al., 2014). Taken together, the current findings demonstrate a great potential to use resident stem cells for vascular regeneration and angiogenesis. The mechanism of stem/progenitor cell differentiation into endothelial or smooth muscle cells may involve in the alteration of biomechanical stimuli, i.e. shear stress and cyclic strain (C. Zhang, Zeng, Emanueli, & Xu, 2013). Accumulating evidence indicates that laminar shear stress and cyclic strain could induce the differentiation of stem/progenitor cells into endothelial or smooth muscle cells, respectively.

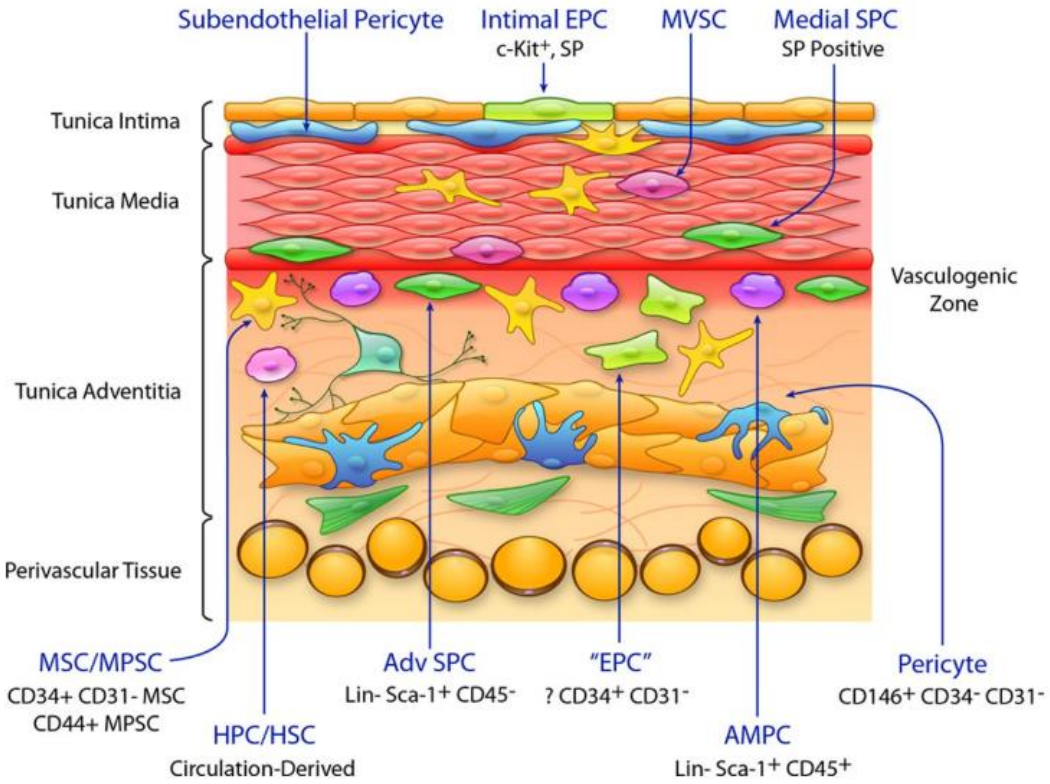


Figure 1.9. Adventitial progenitor cells. Various vascular wall progenitor

cells were discovered within the mural layer of veins or arteries, for instance, Sca-1^{+} smooth muscle progenitors, Sca-1^{+} CD45^{+} macrophage progenitors in mice, CD34^{+}CD31^{-} mesenchymal stem cell and CD44^{+} multipotent stem cells in humans (Peter J Psaltis & Robert D Simari, 2015).

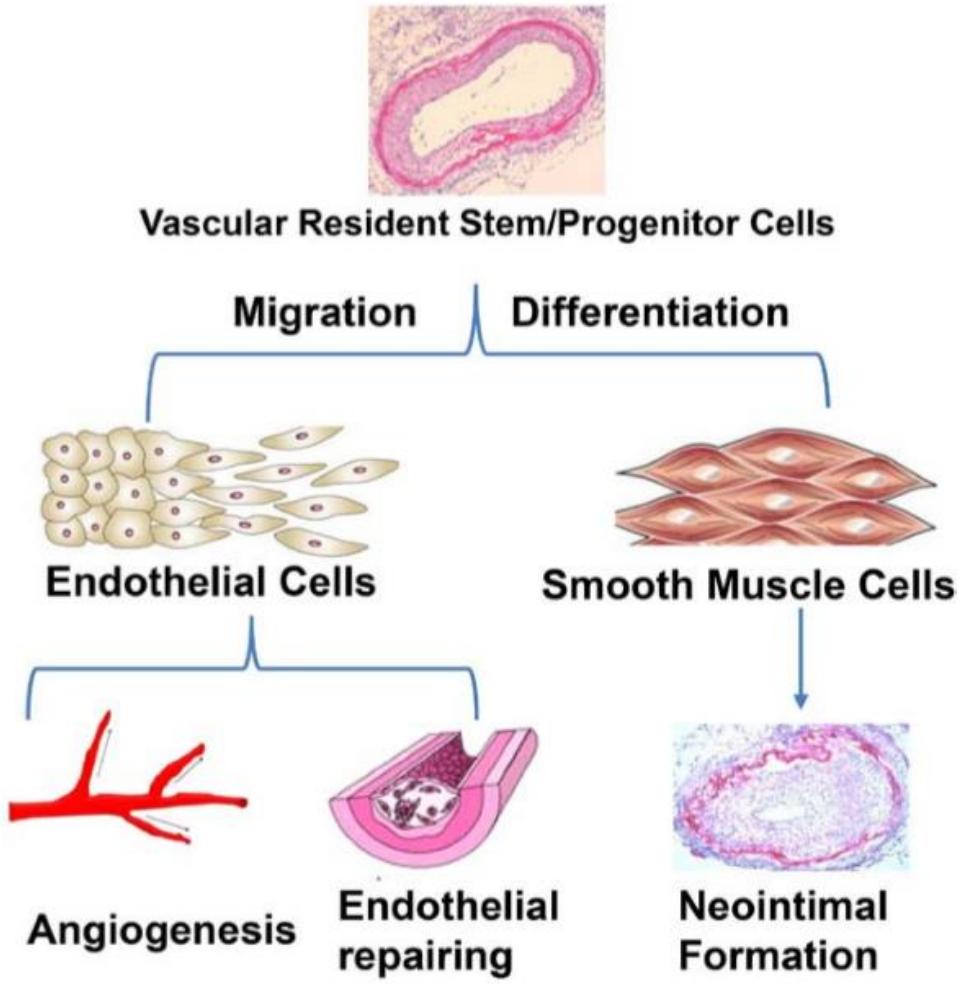


Figure 1.10. Vascular resident stem/progenitor cells were discovered in all 3 layers of vessels. In injured blood vessels, vascular stem/progenitor cells are capable of migrating into the damaged area in response to various stimuli. The cells can then differentiate either into endothelial or smooth muscle cells, respectively, depending on the profile of different cytokines released. The migration and differentiation of vascular stem/progenitor cells contributes to angiogenesis, endothelial repair, and neointimal formation (Xie, Fan, & Xu, 2016).

Table 1.1 Summary of published adventitial progenitor cells (Yanhua Hu & Xu, 2011)

Publications	Species	Location of progenitors	Differentiation potential	Cell markers
(Y. Hu et al., 2004)	apoE$^{-/-}$ mice	Adventitia	SMC	Sca-1
(Howson et al., 2005)	Rat aorta	Various sources	Pericytes	CD34/Tie2, NG2, nestin, PDGFR
(Elvin Zengin et al., 2006)	Human vessels	Media and adventitia	EC, hematopoietic and immune cells	CD34, VEGFR2, Tie-2
(Pasquinelli et al., 2007)	Human thoracic aorta	Media and adventitia	EC	CD34, C-kit
(Hoshino, Chiba, Nagai, Ishii, & Ochiai, 2008)	Human pulmonary artery	adventitia	Adipogenic, osteogenic	Vimentin, Collagen I, CD29, CD44, CD105
(Passman et al., 2008)	Mouse embryonic/adult arteries	Media and adventitia	SMC	Sca-1
(Pasquinelli et al., 2010)	Human arteries	Media and adventitia	Adipogenic, chondrogenic, leiomyogenic	Oct-4, STRO-1, Sca-1, NOTCH-1
(Campagnolo et al., 2010)	Human saphenous vein	Various sources	Pericyte	CD34
(Fang, Li, Song, & Li, 2010)	Human fetal aorta	Various sources	EC, SMC, osteogenic and adipogenic	CD105, FLK

1.5. Treatment of Atherosclerosis

A healthy lifestyle is always the best treatment for atherosclerosis. Besides, recommended treatment of atherosclerosis includes but not limited to a plethora of drugs, coronary artery bypass grafting and percutaneous transluminal coronary angioplasty (PCI).

Various medications were invented to retard or even reverse the process of atherosclerosis. Cholesterol medications, or known as statins and fibrates, are able to substantially lower the concentration of low-density lipoprotein (LDL) cholesterol, preventing the arteries from the buildup of fatty deposits. Meanwhile, the upregulation of high-density lipoprotein (HDL) cholesterol by the application of cholesterol medications may also help mitigate atherosclerosis. Anti-platelet medications such as aspirin could be prescribed to reduce the chance of forming a blood clot and blockage. Angiotensin-converting enzyme (ACE) inhibitors slow down the atherosclerosis by reducing the blood pressure as well as establishing other beneficial effects on vascular system. Beta blocker medications are capable of reducing heart rate and blood pressure which decrease the demand of patient's heart and relieve the symptoms of chest pain. Other medications include but not limited to calcium channel blockers, water pills and specific medications for certain complications of atherosclerosis.

Surgical procedures are required once patients have severe symptoms of atherosclerosis. For angioplasty and stent placement, a long, thin catheter is inserted into the narrowed part of patient's artery. A second catheter containing a deflated balloon is subsequently delivered into the narrowed area of the artery. The balloon is then inflated. The stent is subsequently expanded, compressing the deposit against the vessel wall and widening the narrowed artery. The stent is made of mesh metal which is usually coated with different drugs to prevent the arteries from re-stenosis. After the surgery, the stent is normally left in the narrowed artery to keep it open (Figure 1.11).

Coronary artery bypass grafting (CABG) is to create a bypass using a vessel from great saphenous vein or internal mammary artery, which allows blood to flow around the blocked or narrowed arteries. Other surgical treatments include endarterectomy and fibrinolytic therapy (Janet M Torpy, Lynm, & Glass, 2004).

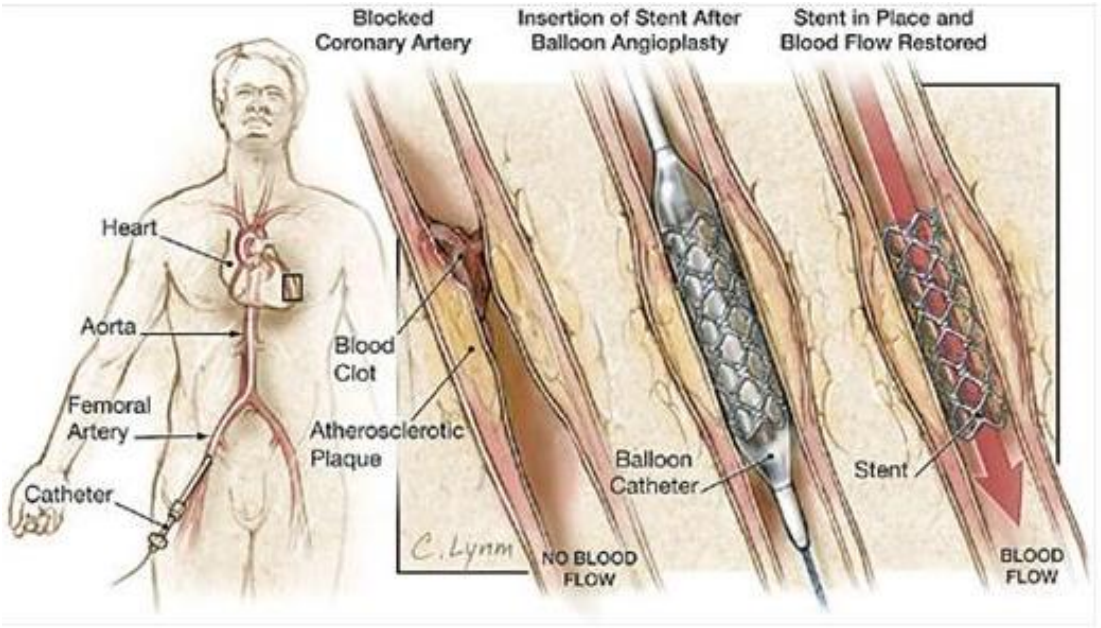


Figure 1.11. Schematic representation of percutaneous coronary intervention (PCI) (J. M. Torpy, Lynm, & Glass, 2008). Catheter with balloon and stent is delivered into narrowed area of coronary artery. Balloon is then inflated and retreated. Stent is left and kept open to support the lumen.

1.5.1.Complications of PCI

Percutaneous coronary angioplasty, as a non-surgical treatment of atherosclerosis, is less invasive compared to the coronary artery bypass grafting (CABG). However, long-term restenosis (Figure 1.12) after PCI surgery brings new challenge for the treatment of atherosclerosis (Nikol, Huehns, & Höfling, 1996).

Restenosis is the recurrence of stenosis. When narrowed blood vessels received treatment such as PCI to clear the blockage and widen the narrowed

artery, restenosis can occur after the surgery, leading to restricted blood flow. Once a stent is applied and restenosis happens, the situation is named in-stent restenosis. If restenosis only occurs after balloon angioplasty, it is named post-angioplasty restenosis (Hamid & Coltart, 2007). Restenosis after PCI is characterized as a distinct pathophysiological process other than atherosclerosis (Costa & Simon, 2005). Inflammation as well as the proliferation of fibroblast and smooth muscle cells plays important roles in restenosis. In human studies, a mural thrombus could be formed at the early stage of atherosclerosis, prior to the invasion of smooth muscle cells, T-lymphocytes cells and macrophages. The implanted stent can be fully covered by this early neointima 4 weeks after the surgery. Extracellular matrix subsequently enhances and smooth muscle cells can be discovered with lymphocytes next to the stent (Toutouzas, Colombo, & Stefanadis, 2004). Late phase of post-angioplasty restenosis involves in the dedifferentiation, proliferation and migration of smooth muscle cells towards intima. These smooth muscle cells are capable of releasing hyaluronan, proteoglycans and collagens, all of which compose the majority of neointimal hyperplasia (Toutouzas et al., 2004). Due to the high risk of post-angioplasty restenosis in patients who received PCI, drug-eluting stents (DES) was hence invented. DES is coated with medications which could prevent the surgical arteries from restenosis. For instance, sirolimus, as an inhibitor of mTOR, can substantially suppress the migration and proliferation of smooth muscle cells, decreasing the extent of restenosis in the arteries with stents (Curcio, Torella, & Indolfi, 2011). Although DES showed a significant improvement in restenosis compared to bare metal stents, there is no marked difference in late stent thrombosis incidence.

Stent thrombosis is defined as a newly formed thrombus in the segment where a previous stent was implanted. The reasons for the occurrence of late stent thrombosis are variable. Stenting across ostia of major arterial branches, exposure to radiation therapy, plaque disruption in non-stented arterials,

extensive plaque prolapse and diffuse in-stent restenosis can all contribute to late stent thrombosis, impairing intimal healing over the stent struts (Farb, Burke, Kolodgie, & Virmani, 2003).

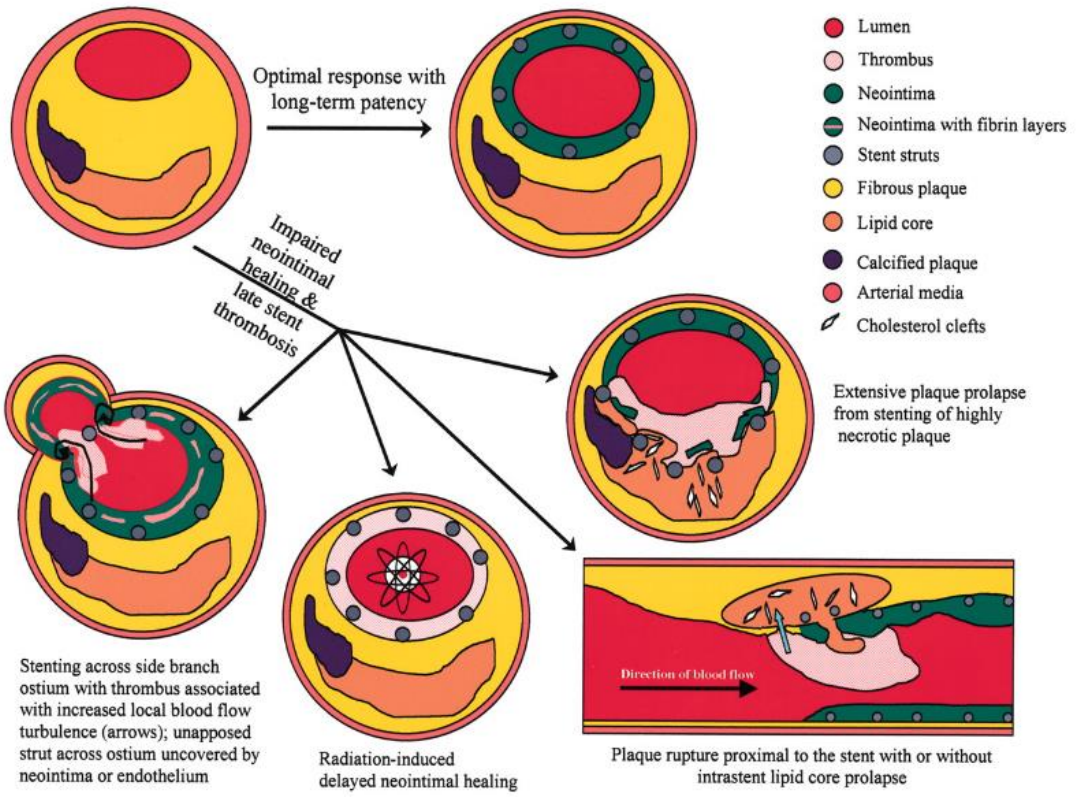


Figure 1.12. Schematic diagram of the postulated pathological mechanism of the development of late stent thrombosis with impaired neointimal healing (Farb et al., 2003).

1.6. Animal Model of the Vascular Disease

The first vascular research with animal models dates back to 100 years ago, which was a rabbit model published by Ignatowski in 1908 (Getz & Reardon, 2012). Knowledge of the pathogenesis and therapy of atherosclerotic disease and the use of animal models in arteriosclerosis research have evolved almost simultaneously. The use of animal models in the study of arteriosclerosis is essential in a set of aspects. For instance, evaluation of a risk factor as a single independent variable, with almost complete exclusion of other factors, can best be performed in animals free of intercurrent diseases or abnormalities and with well-known genetic characteristics (Weiss, Kools, & Taylor, 2001). On the other hand, the role of vascular injury due to angioplasty, alloimmune responses or vein grafts can be investigated alone or in combination with other factors that either aggravate or have beneficial effects(Xu, 2004). Furthermore, experiments using animals are the only way to develop and test new diagnostic, preventive and therapeutic procedures for both ethical and practical reasons. The investigator can choose the species, time and method, and obtain samples of both tissue and serum as well, as these methods would be difficult, if not impossible, in studies with human subjects.

Rabbit is the first and one of the most frequently used animal at all points of arteriosclerosis research. Lesions analogous to those in humans have been induced in the rabbit by a variety of methods. Since rabbits are very susceptible to a cholesterol-enriched diet, hypercholesterolemia and atherosclerotic lesions in the intima of large arteries can be obtained shortly after administration of the food. The Watanabe heritable hyperlipidemic (WHHL) rabbit, which is an excellent model of homozygous familial hypercholesterolemia in humans, has also been used.

Due to well-defined genetic systems of transgenic and knockout mice, a number of investigators have used the mouse as an experimental system for

arteriosclerosis research (Weiss et al., 2001). Hundreds of inbred lines have been established, and the genetic map is relatively well defined, and both congenic strains and recombinant strains are available to serve better the genetic experimentation. In just a few years, murine lipoproteins have been characterized, genetic variants of apolipoproteins identified, and genetic variation in susceptibility to atherosclerosis among inbred mouse strains demonstrated (Carmeliet et al., 1997; Ivan et al., 2002; Roque et al., 2000). In addition, reduced experimental animal numbers can be achieved due to inbred strains having low variability, which is also of economic benefit. The mouse is becoming a widely used model for studying all aspects of arteriosclerosis, and thus, this chapter will discuss all types of mouse models for vascular disease, highlight the usage of rat, rabbit and pig models and attempt to update progress in the use of animal models for studies on vascular diseases.

1.6.1. Mouse Model of Atherosclerosis

Atherosclerosis is a chronic cardiovascular disease which can remain asymptomatic for decades. It is also the leading cause of stroke, myocardial infarctions and other vascular diseases, of which mechanism is still not fully understood. Animal models are designed to be preliminary tools for a better understanding of the pathogenesis, improvement in diagnosis, prevention, and therapy of vascular diseases in humans. Animal models are easily manageable, as compounding effects of dietary and environmental factors can be controlled experimentally. Blood vessel samples can be taken for detailed experimental and biomolecular examination. A thorough understanding of the animal models is necessary and complete analysis must be validated so that the data can be extrapolated to humans. Attracted by the well-defined genetic systems, a number of investigators have begun to use the mouse as an experimental system for arteriosclerosis research. The

specific advantages and disadvantages of using a mouse model are listed below (Table 1.2). Since the vascular disorder is a complicated disease, which includes spontaneous (native) atherosclerosis, transplant arteriosclerosis, vein graft atherosclerosis and angioplasty-induced restenosis, several models for studying all types of vascular disease have recently been established. Using these animal models, much knowledge concerning the pathogenesis of the illness and therapeutic intervention has been gained.

Table 1.2. Advantages and disadvantages of mouse models

Advantages	Disadvantages
Small, easy to raise, handle and perform surgery.	No naturally occurring atherosclerosis.
Large groups.	Few biological samples (small).
Ease of genetic modification.	Technical difficulties (small).
Low cost of purchase and maintenance.	Unstable atherosclerosis.
Many well-established mouse models to induce atherosclerosis.	Different sites of atherosclerosis and aneurysms.
Minimal amounts of drugs or compounds are needed due to small size.	Different lipid profile compared to humans.
Short lifespan.	
Fully recognized genome.	

1.6.2.Diet-Induced Atherosclerosis

In the 1960s, Wissler used a high-fat diet to induce atherosclerosis but the diet was toxic to mice (Vesselinovitch & Wissler, 1968). Then Paigen modified the formula, however, it still had disadvantages (Paigen, Morrow, Brandon, Mitchell, & Holmes, 1985). Even after modification, diet-induced atherosclerosis in mice was not as obvious as that in larger animals. The

mouse is naturally resistant to atherosclerosis and the lesions in mice are very small and limited to the early fatty-streak stage. In 1992, great progress was made by genetic manipulation. With the help of genetic techniques, apoE$^{-/-}$, LDL receptor-deficiency (Ldlr$^{-/-}$) mice as well as other genetic mouse models were created to induce more human-like atherosclerotic lesions (Piedrahita, Zhang, Hagaman, Oliver, & Maeda, 1992). Numerous animal models based on transgenic mice were then invented to mimic various kinds of pathological states in human.

Mice and human have different lipid profiles, limiting the use of mouse models in medical research. The primary circulating cholesterol in mouse is high-density lipoprotein, and it does not have cholesterol-ester transfer protein. Also, the mouse hardly absorbs dietary cholesterol. This is the reason that wild type mice are usually resistant to atherosclerosis. Therefore, scientists have used genetic engineering to overcome this limitation. Now, both apoE$^{-/-}$ and Ldlr$^{-/-}$ mice are widely used in the investigation of a variety of vascular diseases (Getz & Reardon, 2012). These mice will not show visible vascular lesions when fed a chow diet (Figure 1.13) but may present lesions following an atherogenic diet. Meanwhile, other genetic-deficient mice, such as hepatic lipase-deficiency and human apoB100 expression models, have also been generated to be used in the atherosclerotic field.

At present, experimental mice are fed in a lower fat diet, and many other kinds of ingredients emerge according to the researchers' requirements. However, the atherosclerosis induced by this method is not stable and reproductive enough. Wild-type mice have naturally a very low concentration of triglycerides and LDL in the serum, but have a very high HDL concentration, which would contribute to reducing atherosclerosis. For instance, feeding Western-type diet which contains 35% fat, 50% carbohydrate, 15% protein cholesterol (0.5 to 1%) and cholic acid (0.1% to 0.5%), mice leads to increase in LDL and triglyceride levels but animals display mild atherosclerosis. The cholic acid in Western-type diet can prevent cholesterol from converting into

bile acid. In addition, the presence of cholic acid helps absorb the fat and cholesterol which increases the level of cholesterol in the circulation (Ando et al., 2005). It is worth noticing that cholic acid also has an effect on inflammation and lipoprotein metabolism, which are the key factors of atherosclerosis. However, in mice heterozygous or homozygous for a disrupted apoE gene and LDL receptor gene, a dramatically increase of atherosclerosis was discovered after feeding an atherogenic diet (S. H. Zhang, Reddick, Burkey, & Maeda, 1994).

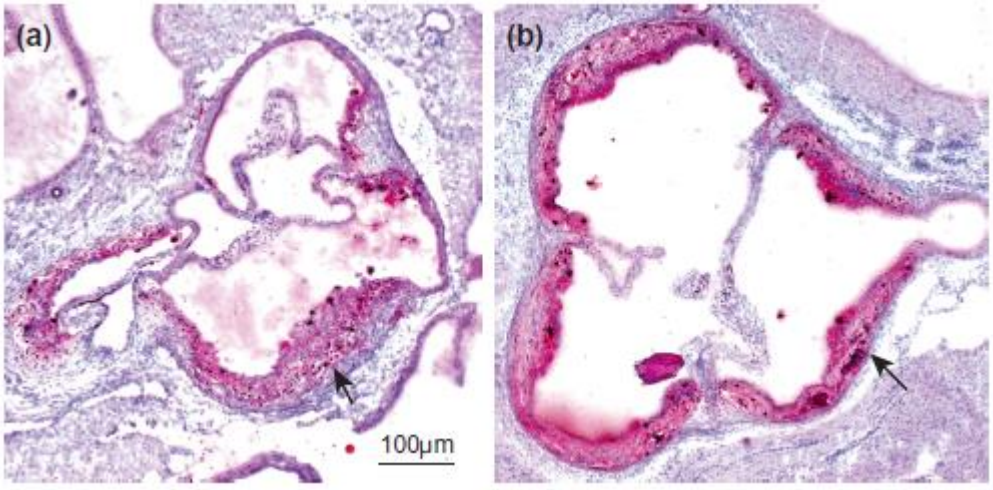


Figure 1.13. Atherosclerosis in the aorta. The image representation of aortic root lesions of atherosclerosis from apoE (a) and LDL (b) deficient mice at the age of 36 months with normal chow diet (Piedrahita et al., 1992).

1.6.3.Mouse Model of Vascular Injury

Spontaneous development of atherosclerosis in experimental animals is time-consuming. Moreover, unlike human whose atherosclerosis mainly happens in coronary arteries, carotid, and peripheral vessels, murine atherosclerosis occur more frequently in aortic arch, aortic root and innominate artery (Getz & Reardon, 2012). By applying local interventions, researchers are able to not only reduce the experimental time but also to select an optimal location and control the extent of the injury. Balloon injury,

guide wire injury, blood flow cessation and stenosis collar were hence developed (Xu, 2004).

Periarterial cuff placement was developed several years ago (Karper et al., 2011). This method allows the structural integrity of the endothelium to be maintained. Typically, under anesthesia, the target artery will be separated from surrounding tissue. A tiny polyethylene cuff will loosely sheath the artery and be tied with a suture. Since the cuff is larger than the vessel, it would not affect blood flow. Then the target artery will be replaced and the wounds will be sutured. Electric injury to the carotid artery of mice can induce vessel injury, in which the artery should be well isolated firstly. A bipolar micro-coagulator can be applied, positioned perpendicularly to the longitudinal axis of the artery to do the injury. A very small current pulse can cause the injury and reducible neointima formation. The blood flow cessation model can be the easiest way to induce vascular injury. Remodeling is carried out with ligation of the target artery, i.e. common carotid artery near its bifurcation. Then the wound is sutured and the mouse is allowed to recover.

1.6.4.Stent Implantation

Stent implantation in a mouse model, along with administration of drugs and chemokines, is a powerful tool to help understand the mechanism and solutions of restenosis caused by a stent implantation in human beings. Under anesthesia, before the isolation of the common carotid artery, a knot is bound around the common carotid artery, 2 knots around the left external carotid artery and last knot can be around the internal carotid artery. The blood flow is stopped by binding the knots on internal and external carotid arteries, and pulling the knot on the common carotid artery. An arteriotomy will be made at the external carotid then a silicon tube containing stent is inserted into common carotid artery to the target position. Later on, pull back the tube by using guide wire and bind the knot on external carotid artery tightly remove

another two knots and close the wound of skin (Simsekyilmaz et al., 2013).

Compared to the mouse model of guide-wire injury, stent implantation mainly focuses on the mechanism of In-stent thrombosis after percutaneous transluminal (coronary) angioplasty (PTCA) with stent implantation in human beings. The development of a miniaturized stent for mouse carotid artery, combined with different knock-out mice allows the study of particular molecular mechanisms for in-stent thrombosis and the tests of various stent-coating drugs. However, the mouse model of guide-wire injury may be more relevant to clinical intra-arterial manipulation but cannot represent the in-stent neointimal formation.

1.6.5. Guide-Wire Injury

Guide-wire injury to the vessel can be achieved by 3 passages of a 0.25mm diameter angioplasty guide wire (Figure 1.14). Under anesthesia, the femoral artery can be separated clearly with the help of a surgical microscope. A suture is inserted through the femoral artery in advance in preparation for further ligation. Then, an arteriotomy can be made, and the guide wire is inserted forward until the aortic bifurcation. The guide wire is then moving back and forth three times to ensure a complete endothelium denudation. After removing the guide wire, the arteriotomy site should be ligated immediately and the wound is sutured (Roque et al., 2000). Firstly applied by Roque et al. in 2000, guide-wire injury model soon became widely accepted as it closely resembled the injury of the endothelium and vessel wall. Typical neointimal hyperplasia undergoes three lesion processes. The first one is a marked loss of smooth muscle cells at an early stage, probably caused by biomechanical stress (Mayr et al., 2000). Next comes to the massive infiltration of mononuclear cells ($CD11b/18^{+}$). Increasing adhesion and chemokines secretion from smooth muscle cells and endothelial cells caused by biomechanical stress and dead cells from early stage may explain the

continuous recruitment of inflammatory cells. At the final stage, cell differentiation, migration and accumulation are involved. Vascular smooth muscle cells mainly constitute the neointimal hyperplasia (Zou et al., 1998).

A major and common complication in vessel injury models is the development of neointimal lesions as early as the 1st week with the peak in 3-4 weeks. Using these models, smooth muscle cell migration and proliferation can be studied. Neointimal lesions usually stop growing in denuding injury models as soon as the luminal surface gets re-endothelialized. Restricting the morphometric analysis to denuded segments assures that all measurements are performed on segments of vessels that have not been influenced by the variable of endothelial regeneration. Unfortunately, not many investigators rigorously control endothelial regeneration and clot-dependent effects on neointima formation. Like the rat balloon injury model, the proliferation of SMC starts in the media within a few days after denudation and if no re-endothelialization occurs, proliferating SMC can be found in the neointima typically within a week. If re-endothelialization occurred before SMC could migrate into the intima medial hypertrophy and hyperplasia will be observed since medial SMC proliferation precedes the arrival of SMC in the intima.

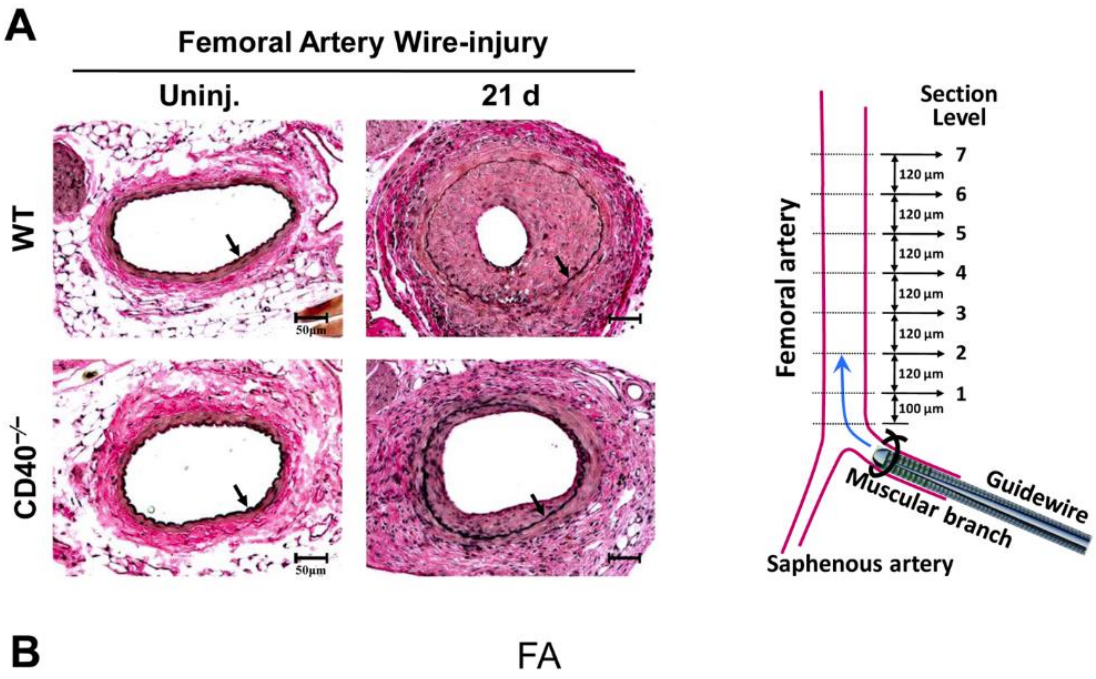


Figure 1.14. Guide-wire injury mouse model. Representative figures of cross sections of guide-wire injury mouse model in wild-type and CD40$^{-/-}$ mice (left panel). Schematic figure of guide-wire injury and angiotomy of femoral artery in mouse (right panel) (Song et al., 2011).

1.7. Adipose Tissue

Adipose tissue is a loose connective tissue which mostly contains adipocytes, as well as a smaller stromal vascular fraction (SVF). SVF consist of adipose-derived stem cells (ADSC), fibroblasts, vascular endothelial cells and immune cells. Adipose tissue was initially thought to be only an inert energy storage organ. However, accumulating studies indicated that adipose tissue is actually the largest endocrine organ, releasing an array of signaling molecules, named adipokines. (Kershaw & Flier, 2004) Adipose tissue is typically classified into two sub-families: brown adipose tissue and white adipose tissue. The latter is capable of secreting adipokines and has an effect on other organs both systematically and locally, which may contribute to various cardiovascular diseases (Kwon & Pessin, 2013).

1.7.1. Perivascular Adipose Tissue (PVAT)

Blood vessels are composed of three different layers: intima, media and adventitia. The perivascular adipose tissue harbors on the adventitia side of almost every blood vessel except for cerebral vasculature (Y.-J. Gao, 2007). Since there is no anatomical barrier between perivascular adipose tissue and the vessel wall, perivascular adipose tissue is regarded as an endocrine organ and has a direct effect on the vasculature (Rajsheker et al., 2010) (Figure 1.15). In 2005, PVAT was proved to release adipokines which had an impact on the proliferation of vascular smooth muscle cells (Barandier, Montani, & Yang, 2005). In 2009, Takaoka demonstrated that implanting abdominal adipose tissue around the blood vessel can increase the neointimal formation after endovascular injury (Takaoka et al., 2009). Taken together, adipose tissue as well as its secretion could have a great impact on vascular remodeling.

There are two types of adipose tissues in human bodies: brown adipose tissue and white adipose tissue. Brown adipose tissue is mainly responsible

for generating heat which is often regarded as good fat, containing much more iron-containing mitochondria and tiny blood vessels than white adipose tissue does (Cinti, 2011). PVAT is a mixture of brown adipose tissue and white adipose tissue. The composition of PVAT varies from region to region. For instance, PVAT surrounding thoracic aorta is mainly composed of brown adipose tissue, while PVAT around abdominal aorta and mesenteric artery is mainly white adipose tissue, indicating their diverse functions in different regions (Frontini & Cinti, 2010; P. Wang et al., 2008).

Perivascular adipose tissue is not only a loose connective tissue, but also an endocrine organ. It was well known that adipocytes can induce the proliferation of smooth muscle cells, but the mechanism was not clear until 2005. Barandier et al. discovered that growth factors present in conditioned medium from mature adipocytes or preadipocytes can induce the proliferation of vascular smooth muscle cells, the effect of which could be diminished by proteinase K (Barandier et al., 2005). Later, Lamers et al. demonstrated that conditioned medium from adipocytes promoted the migration of vascular smooth muscle cells (Lamers et al., 2012). Numerous studies focused on the relationship between the characteristics of PVAT and obesity. One research revealed that PVAT from obese or aged mouse could significantly enhance the proliferation of vascular smooth muscle cells, indicating that PVAT's features may vary according to donors' body weight and age. This difference may be caused by the different composition of adipokines released by the donors' PVAT which subsequently influenced the vasculature locally or systematically (Barandier et al., 2005).

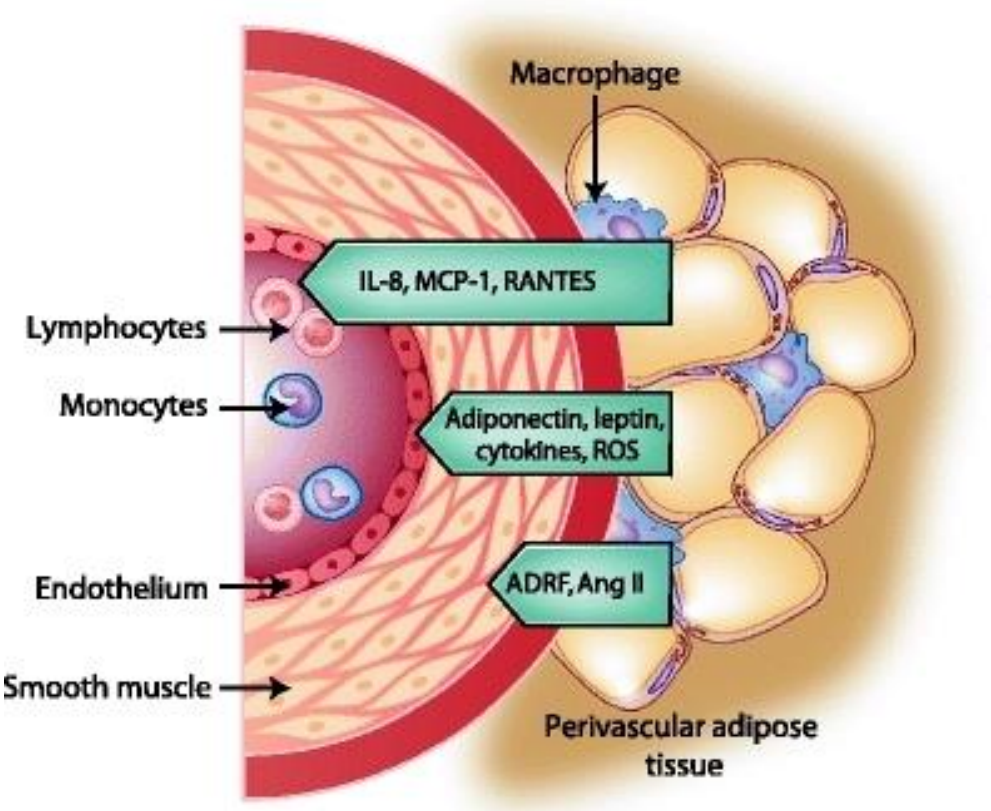


Figure 1.15. Interaction between perivascular adipose tissue and vessels (Meijer et al., 2011). Perivascular adipose tissue could secret various adipokines such as adiponectin and leptin, which could directly participate in vascular remodelling with endothelium, smooth muscle cells, monocytes, lymphocytes and other mediators involved. ADRF: adventitia-derived relaxing factor; Ang II: angiotensin II; IL8: interleukin 8; MCP-1: monocyte chemoattractant protein 1; ROS: reactive oxygen species.

1.8. Chemokine and Adipokine

Chemokines belong to a family of tiny cytokines which are secreted by cells, inducing chemotaxis of nearby cells. They have a relatively low molecular weight between 8 to 12 kDa, some of which are able to stimulate the recruitment of leukocytes (Mélik-Parsadaniantz & Rostène, 2008). All the chemokines share a similar genomic, consisting of three exons and two introns (Groves & Jiang, 1995). Chemokines have been classified into four subfamilies according to the spacing of their first two cysteine residues: CXC, CC, CX3C and XC (Mélik-Parsadaniantz & Rostène, 2008). Chemokine receptors belong to a seven transmembrane G-protein coupled receptor family with a short, acidic N-terminal end, three intracellular, three extracellular hydrophilic loops, and an intracellular C-terminus (Murdoch & Finn, 2000). Chemokine receptors transduce cell signaling after ligand binding, promoting various signaling pathways to generate the migratory effect.

Adipokines belong to a family of chemokines which are exclusively secreted by adipose tissue. They share the same characteristics as other chemokines. Leptin was the first adipokine discovered in 1994, opening a new perspective in many fields including the cardiovascular research. Later, other adipokines, such as adiponectin, visfatin and resistin were successively identified. Adipokines are mainly released by white adipose tissue, and can induce various physiological or pathological activities. For instance, in obese individuals, plasma leptin is higher than that in the healthy individuals. A higher concentration of leptin was believed to promote inflammatory process whereas adiponectin was regarded as a protective role in inflammation (Kwon & Pessin, 2013).

1.8.1. Leptin and Its Role in Vascular Remodeling.

Leptin is a 16 kDa peptide hormone responsible for long-term regulation of

energy balance. Firstly discovered in the hypothalamus in 1994, leptin opened a new gate to study the role of adipose-derived chemokines or so called adipokines (Y. Zhang et al., 1994). Elevated leptin concentration in circulation strongly correlates with obesity (or body mass index), hyperinsulinemia and insulin resistance. Body weight loss could decrease the concentration of serum leptin but how body absorbs or expels serum Leptin mechanically was still unknown. Leptin was previously known to play roles in the regulation of food intake and energy expenditure, but increasing studies have demonstrated its additional effects on the cardiovascular system (Figure 1.16), where widespread distribution of leptin receptor (OBR) has been identified (Sweeney, 2002). Leptin may contribute to atherosclerosis through activation of various mechanisms, including endothelial dysfunction (Lembo et al., 2000), lipid metabolism (Lundåsen, Liao, Angelin, & Rudling, 2003; Maingrette & Renier, 2003), proinflammatory effect (Shamsuzzaman et al., 2004) and proliferation of smooth muscle cells (Beltowski, 2006; Parhami, Tintut, Ballard, Fogelman, & Demer, 2001). Shan J et al discovered that leptin stimulated proliferation of murine smooth muscle cells (SMCs) *via* the mTOR-signaling pathway, which may contribute to enhancing neointimal hyperplasia in obese humans (Shan et al., 2008). Deletion of either leptin or OBR in ob/ob (leptin-deficient) or db/db mice significantly mitigated the formation of neointima (Schäfer et al., 2004). The mechanism of leptin-induced neointimal formation after guide-wire injury in the femoral artery is believed independent of blood pressure and energy balance (Bodary et al., 2007). In addition, leptin may participate in vascular remodeling and stiffness by altering extracellular matrix production in VSMC through the PI3K/Akt pathway (Martínez-Martínez et al., 2014). Taken together, leptin can have an effect on vascular remodeling.

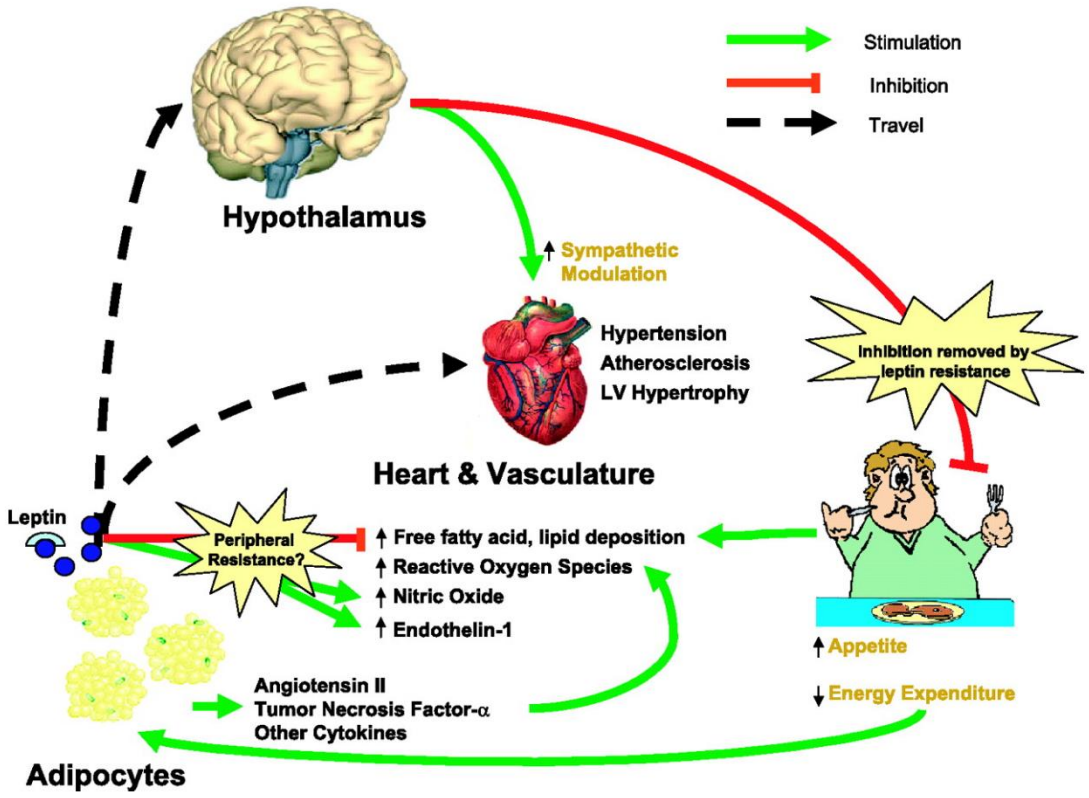


Figure 1.16. The systemic effect of enhanced serum leptin on the cardiovascular system (Yang & Barouch, 2007). Obese individuals have higher concentrations of leptin, which may contribute to various diseases of heart and vasculature *via* different mechanisms. Selective central leptin resistance leads to hypertension, atherosclerosis and LVH.

The pleiotropic characteristics of leptin owe to the ubiquitous distribution of leptin receptors (OB-R). Leptin receptor belongs to a family of transmembrane receptor which has a similar structure to class 1 cytokine receptor family, such as LIF, IL-2, and IL3 etc (Hegyi, Fülöp, Kovács, Tóth, & Falus, 2004). OB-R has six spliced forms: OB-Ra, OB-Rb, OB-Rc, OB-Rd, OB-Re and OB-Rf (Lee et al., 1996). Six spliced forms are classified into three groups: short (OB-Ra, OB-Rc, OB-Rd, OB-Rf), long (OB-Rb), secreted (OB-Re). Short isoforms is also capable of transducing signaling pathways via JAK but their activities are substantially much weaker than those induced by long isoforms (Bjørbæk, Uotani, da Silva, & Flier, 1997). Li et al addressed

the role of OBRa by deleting the OBRa-specific exon in mouse model. These mice displayed the phenotype with decreased fasting blood glucose and an improved glucose tolerance. OBRa overall showed a similar role compared to OBRb but with a limited control of leptin metabolic effects. The expression of OBRc is either as abundant as or more prevalent than OBRa in various tissues, such as cortex, hippocampus and lung, indicating that OBRc may be at least as important as OBRa in these tissues in mediating the effect of leptin. Interestingly, when the expression of OBRb is discovered, engagement of OBRb into heteromers with short isoforms of leptin receptors is likely to occur, implying the interactions of OBRb versus the short isoforms (Dam & Jockers, 2013). OBRe, as a soluble leptin receptor in blood, has been reported secreted by adipose tissue alone or in combination with leptin. Serum leptin and OBRe has an inverse proportion during the diurnal variation. In leptin-deficient humans, OBRe could bind to leptin in the serum, exhibiting the biological activity of leptin (Gavrila et al., 2003). Therefore, only OB-Rb is considered as the functional receptor (Tartaglia et al., 1995) (Figure 1.17).

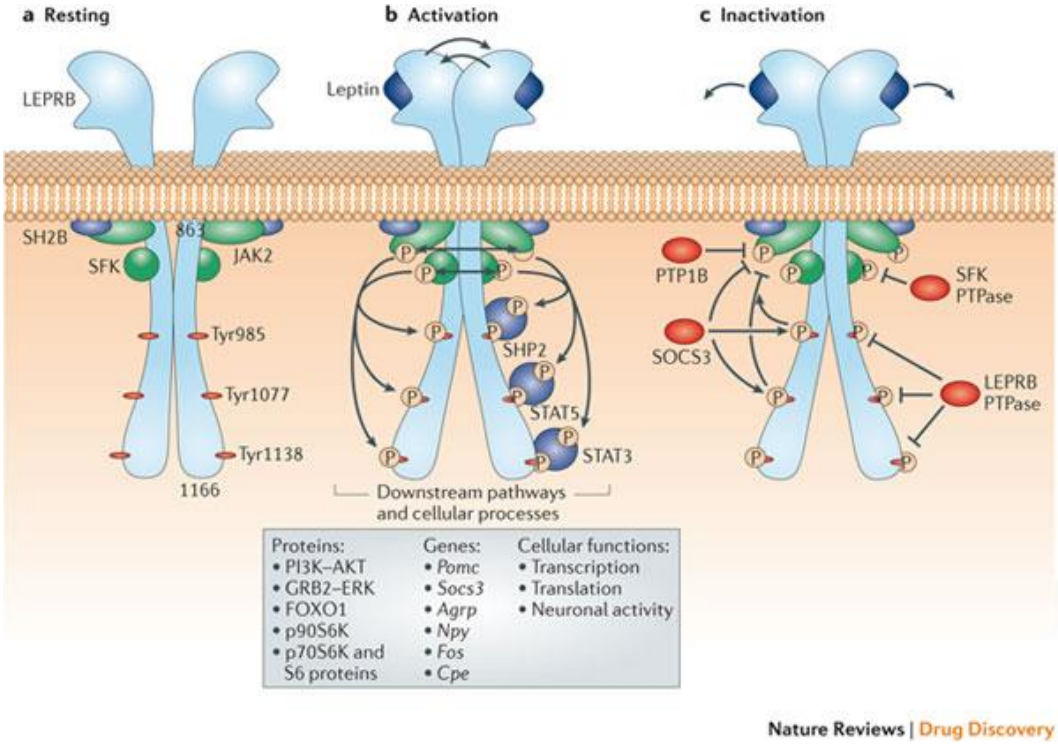


Figure 1.17. The structure of leptin receptor b in resting, activation and in-activation stages (Coppari & Bjørbæk, 2012). a, the resting stage of the long isoform of the leptin receptor b which exists as a homodimer at the plasma memberane. **b**, when leptin binds to leptin receptor, the two receptor subunits undergo the changes of conformation, leading to the transphosphorylation and transactivation of JAK2 and SFK protein. Subsequently, activated JAK and SFK phosphorylate Tyr residues of leptin receptor b. **c**, leptin receptor b is inactivated by protein tyrosine phosphatase 1B (PTP1B), followed by the inhibition of JAK2 *via* SOCS3.

1.8.2. Adiponectin

Adiponectin is a 244-amino-acid-long polypeptide exclusively released by adipose tissues. Identified by different methods from four groups, adiponectin was also named GBP-28, apM1, AdipoQ and Acrp30 (Maeda et al., 1996). The concentrations of plasma adiponectin and body weight of individuals have an inverse relationship. Massive evidence suggested that adiponectin is a beneficial adipokine with anti-diabetic, anti-inflammatory and anti-atherogenic effect (Matsuzawa, 2010).

Adiponectin mainly binds to three receptors: adiponectin receptor 1 (ADIPOR 1), adiponectin receptor 2 (ADIPOR 2) and T-cadherin. ADIPOR1 and 2 are G protein-coupled receptors (GPCRs), possessing seven transmembrane domains. Activation of these two receptors mediates increased AMPK and PPAR-α ligand activity. One group revealed that adiponectin had anti-atherogenic and anti-inflammatory properties during the development of atherosclerosis (Arita et al., 2002). For instance, the expression of adiponectin was greatly increased in sub-intimal area after the vascular endothelium injury (Okamoto et al., 2000). Elevating concentration of adiponectin significantly reduced the adhesion of monocyte and reduced the expression of E-selectin, VCAM-1 and ICAM-1 on the endothelium (Ouchi et

al., 1999), both suggesting that adiponectin had a protective role against neointimal formation.

1.9. Leptin-Induced Signaling Pathways

1.9.1. Leptin Receptor B (OBR)

As mentioned previously, the leptin receptor codes for six leptin receptor isoforms *via* RNA splicing. All of them have an extracellular domain which can bind to leptin but only leptin receptor b contains a full-length intracellular domain, which is necessary for signal transduction cascade (Friedman & Halaas, 1998). The action of OBR is commonly induced by JAK/STAT (Zabeau et al., 2003), AMPK (Minokoshi et al., 2002), PI3-Akt and MAPK (Frühbeck, 2006) pathways, which can be inhibited by cytokine signaling 3 receptors (SOCS3) (Frühbeck, 2006) (Figure 1.18).

1.9.2. JAK/STAT Signaling Pathway

The JAK-STAT pathway transmits the signal from outside the cell into gene promoters inside the nucleus, which is the main signaling alternative to second messenger system. Leptin receptor b structurally binds to Janus kinase family of tyrosine kinases (JAK2). Once stimulated by leptin, leptin receptor b starts dimerization, leading to its activation and auto-phosphorylation of JAK2. In the meantime, JAK2 also phosphorylates leptin receptor b thus activating various downstream signaling pathways (Banks, Davis, Bates, & Myers, 2000). Leptin receptor b contains four tyrosine residues (Tyr^{974}, Tyr^{985}, Tyr^{1077} and Tyr^{1138}) and the latter three are closely related to JAK phosphorylation. Replacement of either one of the three residues or combinations of them caused leptin resistance and obesity, suggesting that all three intracellular tyrosine residues are required for downstream activation (Hekerman et al., 2005).

For STAT3 pathways, leptin mainly stimulates STAT3 but can activate STAT1, -5, -6 as well. Tyr^{1138} is required for activation of STAT1 and STAT3 (Münzberg, Björnholm, Bates, & Myers Jr, 2005). Replacing Tyr^{1138} residue with a serine residue leads to inactivation of STAT3 without affecting the

activation of other STAT family members. In response to leptin, STAT3 binds to phospho-Tyr1138, allowing JAK2 to phosphorylate and activate STAT3 (Morris & Rui, 2009). SOCS protein was thought to be the negative-feedback of JAK/STAT pathway. With a SH2 domain, SOCS can be induced by a wide range of cytokines, serving as a negative regulator (Müller, Kuttenkeuler, Gesellchen, Zeidler, & Boutros, 2005). The knockdown of SOCS3 by RNA interference greatly enhanced the tyrosine phosphorylation of JAK2 and STAT3 (Dunn et al., 2005).

Brief leptin treatment could stimulate phosphorylation of STAT3, which had peak activation after 30 to 60 minutes in human embryonic kidney (HEK) 293 cells, declined to 80% of peak level at 4 hours after the treatment and remained at 60% of peak level at 24 hours after the treatment. However, phosphorylation JAK2 was more transient and dropped quickly to 40% to 50% of peak level at 1 to 4 hours after the treatment and below 30% after 8 hours in human embryonic kidney (HEK) 293 cells. In addition, SOCS3 was undetectable before leptin treatment, but can be detected 8 hours after leptin treatment by Western blotting, suggesting SOCS3 was an important feedback mechanism for JAK/STAT signaling (Dunn et al., 2005).

1.9.3.MAPK (mitogen-activated protein kinase) Cascade

The MAPK pathway can be stimulated *via* either leptin receptor a or b but the activation is much stronger when induced by leptin receptor b. Leptin triggers MAPK pathway in two different ways: JAK2 receptor-dependent activation and independent phosphorylation, both of which need an intact catalytic domain of SHP-2 (Bjørbæk et al., 1997).

Activation of MAPK pathways can be observed both *in vivo* and *in vitro*, participating in many pathological and physiological processes. For instance, the phosphorylated ERKs led to the expression c-Fos and Egr-1 which were related to cell proliferation and migration. In osteoplastic precursor cells, it has

been concluded that leptin induced cell apoptosis *via* MAPK cascade through ERK 1/2 activation of cytosolic phospholipase A, which inversely contributed to cytochrome c release and the induction of caspase3 and caspase 9 (G. S. Kim et al., 2003). Besides ERK 1/2, leptin could activate other members of MAPK family such as p38 MAPK pathway. In vascular smooth muscle cells, leptin reportedly led to hypertrophy *via* p38 MAPK pathway, indicating a potential relationship between leptin and vascular remodeling (Mayr et al., 2000).

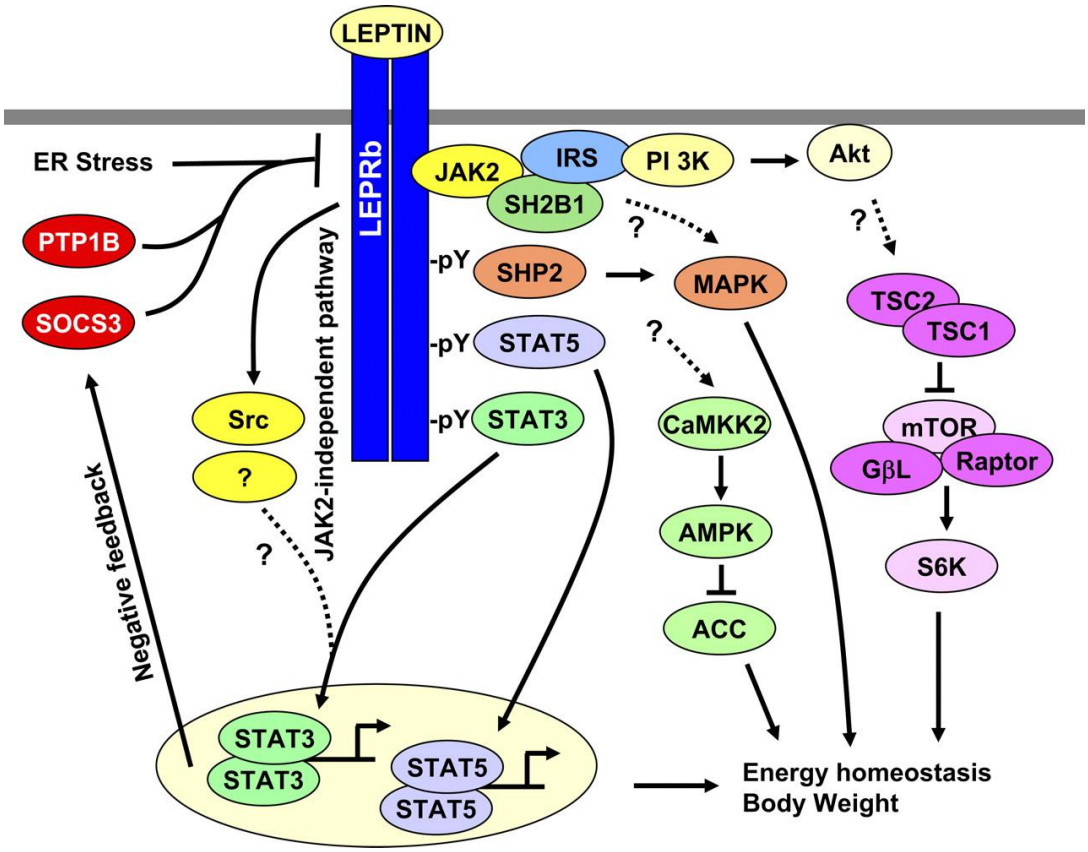


Figure 1.18. Well-established signaling pathways induced by leptin receptor b (Morris & Rui, 2009). Leptin binds to leptin receptor b, activating the phosphorylation of LEPRb-associated JAK2 on $Tyr^{985/1077/1138}$. SHP2 binds to Tyr^{985} which is related to the activation of MAPK pathway. Suppressor of cytokine signaling-3 (SOCS3) also binds to Tyr985 to inhibit leptin signaling as a negative feedback. STAT 3 and 5 are phosphorylated by the activation of JAK on Tyr^{1077} and Tyr^{1138} respectively.

1.10. GTPase

Various intracellular signaling pathways have been identified during cell migration which includes MAPK cascades, Ser/Thr and Thy kinases, scaffold proteins, etc. However, one group of particular families of proteins seems most relevant to cell migration, the GTPases. Small GTPases are a family of hydrolase enzymes which can catalyze and hydrolyze guanosine triphosphate (GTP) to guanosine diphosphate (GDP). The activation of GTPases is strongly linked to cell proliferation, differentiation, migration, and lipid vesicle trafficking. Among all 20 members of GTPases family, three GTPases, Rac, Rho and Cdc42, play crucial roles in coordinating cytoskeleton during cell migration (Xin Zhou, Herbst-Robinson, & Zhang, 2012) (Figure 1.19, cell type not mentioned in the review). Tissue culture studies in fibroblast showed that Rho was involved in assembly of myosin filaments which was a contractile actin. However, Rac and Cdc42 were mainly related to the polymerization of actin to form lamellipodium and filopodia respectively (Raftopoulou & Hall, 2004). Additionally, all three GTPases facilitate the matrix adhesion complexes as well as affect the microtubule cytoskeleton and gene transcription (Etienne-Manneville & Hall, 2002).

Cell migration is achieved by the extension of lamellipodium in the front, followed by the contraction of cell body and the detachment of adhesion site at the rear. Rac and Cdc42 are indispensable in the front to regulate actin polymerization and lamellipodium. The highest concentration of active Rac in the front leading edge can be detected in the migrating fibroblasts (Kraynov et al., 2000). The major target of Rac for the stimulation of lamellipodial extension is WAVE proteins. On the other hand, Cdc42 mainly binds to WASP proteins, activating the Arp2/3 complex to trigger actin polymerization (Welch & Mullins, 2002). WAVE/WASP can bind to GAPs and GEFs, hence generating positive or negative feedback to regulate the extent of actin polymerization (Ridley et al., 2003). Chemotaxis requires extracellular cues

which are regulated by Cdc42. For instance, once Cdc42 was inhibited in macrophages, the cells displayed a random migration. What is more, if Rac activity was blocked, the macrophages did not migrate anymore, indicating that Cdc42 directs Rac activity in the cell front (Etienne-Manneville & Hall, 2002). Unlike Rac and Cdc42 mainly acting in the front, Rho was believed to control the contraction and retraction forces at the rear, which was also associated with focal adhesion assembly. Also, the distribution of Rho activity during the cell migration displayed an inverse pattern to Rac and Cdc42 (Raftopoulou & Hall, 2004). Activation of Rac in the front edge could suppress Rho activity. Meanwhile, the activation of Rho at the sides and rear would suppress Rac activity, indicating different roles of Rac, Cdc42, and Rho during cell migration. Inhibition of Rho resulted in an extended tail due to the decreased ability of actomyosin-based contractility. The underlying mechanism of Rho during cell migration was hence developed. Myosin filament assembly is considered as a major target of Rho, which is activated by Ser/Thr kinase p160ROCK (Alblas, Ulfman, Hordijk, & Koenderman, 2001). Phosphorylation of 160ROCK is responsible for the phosphorylation of LIM kinase, resulting in the phosphorylation of cofilin and myosin light chain, leading to the F-actin stabilization and myosin crosslinking (Raftopoulou & Hall, 2004).

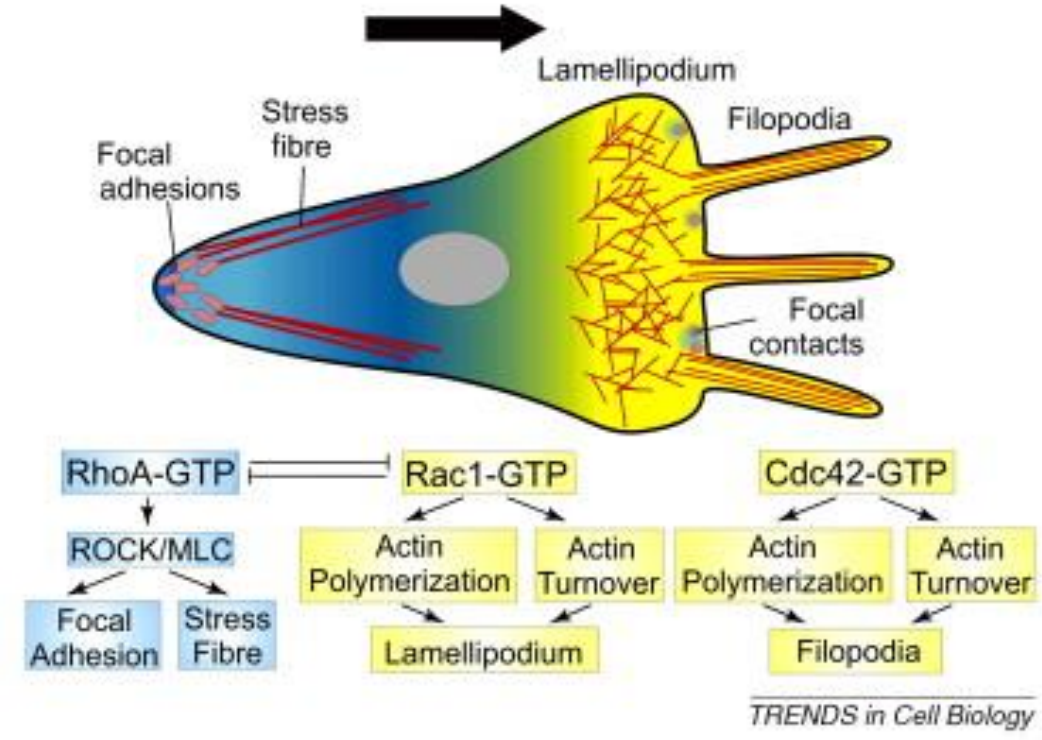


Figure 1.19. Representative figure of cell polarity with the activation of relevant GTPases families. Cell migration depends on cell polarity with cell protrusions such as filopodia and lamellipodia in the front and focal adhesion at the rear. GTPases family plays an important role in cell polarity. RhoA is mainly involved in cell contraction at the back. Cdc42 is responsible for controlling the actin cytoskeleton in filopodia. Rac helps the formation of lamellipodia. However, recent studies showed that all three GTPases family could be activated at both front and rear (Mayor & Carmona-Fontaine, 2010).

1.11. Hypothesis and Aims of Study

Obesity is associated with a significantly higher risk of cardiovascular disease (Korner & Aronne, 2003). The expansion of adipose tissue in obese individuals is closely linked to the secretion of plasma adipokines, which were originally thought only to be related to energy homeostasis (Lehr, Hartwig, & Sell, 2012). Among all adipokines, including adiponectin, visfatin, and resistin, leptin was firstly discovered in 1994 (Conde et al., 2011). Obese individuals strongly correlate with higher levels of plasma leptin, a peptide hormone, mainly secreted into the circulation by white adipose tissue (Schulze & Kratzsch, 2005). Accumulating articles demonstrated that leptin has various biological effects on cell migration, proliferation and differentiation (Beltowski, 2006; Lembo et al., 2000; Parhami et al., 2001; Schäfer et al., 2004; Schroeter et al., 2008).

Recent studies have shown that a range of multipotent stem/progenitor cells exist in the adventitia of the vascular wall (Majesky, Dong, Hoglund, Mahoney, & Daum, 2011; E. Torsney & Q. Xu, 2011; Xie et al., 2016). Our laboratory identified the presence of adventitial progenitor cells, which are positive for Sca-1 and CD34 (Y. Hu et al., 2004). This heterogeneous population of cells can give rise to different cell lineages, including SMCs (Y. Chen et al., 2013; M. M. Wong et al., 2013), endothelial cells (Wong et al., 2014; E. Zengin et al., 2006) and macrophages (P. J. Psaltis et al., 2014; P. J. Psaltis & R. D. Simari, 2015), which may contribute to neointimal formation (Xie et al., 2016). Considering the positive correlation between plasma leptin and cardiovascular disease, several laboratories have investigated biological effects of leptin in the cardiovascular system. However, little is known about whether leptin exerts an effect on adventitial progenitor cells.

Taken together, we postulate that **leptin can aggravate neointimal formation in mouse vascular injury models by inducing migration of Sca-1$^+$ adventitial progenitor cells.**

Chapter 2
Materials & Methods

2.1 Materials

2.1.1 Culture Medium, Recombinant Proteins, Inhibitors and Antagonist.

Table 2.1 Culture medium, recombinant proteins, inhibitors and antagonists

Name	Company	Application
DMEM	ATCC 302002	Cell culture
DMEM	Invitrogen 11960085	Cell culture
FBS	GIBICO 10270	Cell culture
LIF	Merck Millipore 1050	Cell culture
2-mercaptoethanol	GIBCO 21985-023	Cell culture
Penicillin	GIBCO 15140122	Cell culture
Streptomycin	GIBCO 15140122	Cell culture
L-glutamine	GIBCO 25030081	Cell culture
EmbryoMax	EMD Millipore ES-009-B	Cell culture
Leptin	Peprotech 450-31	Cell stimulation
Adiponectin	Peprotech 315-26	Cell stimulation
Visfatin	Abcam, ab62617	Cell stimulation
Resistin	Peprotech 450-28	Cell stimulation
Leptin antagonist	Prospec, CYT354	Receptor inhibition
STAT3 Inhibitor	Merckmillipore, WP1066	Pathway inhibition
ERK inhibitor	Cell signaling, PD98059	Pathway inhibition

2.1.2 Antibodies

Table 2.2 Antibodies.

Name	Company	Application
Anti-Sca-1-FITC	Abcam, ab25031	FACS, IF
Anti-CD45-APC-Cy7	Biolegend, 103115	FACS, IF
Anti-CD29-PE	BD Pharmingen, 562801	FACS, IF
Anti-CD11b-BV785	Biolegend, 101243	FACS
Anti-CD31-BV510	BD Horizon, 563089	FACS, IF
Anti-CD140a-APC	eBioscience, 17-1401-81	FACS
Anti- pJAK2	Cell signaling, 3771S	WB
Anti- JAK2	Cell signaling, 3230	WB
Anti- pSTAT3	Cell Signaling, 9131S	WB
Anti- STAT3	Cell signaling, 4904	WB
Anti- pERK 1/2	Santa Cruz, sc-16982-R	WB
Anti- ERK 1/2	Cell Signaling, 4695P	WB
Anti- pOBR	Invitrogen, PA5-64638	WB, IF
Anti- OBR	Invitrogen, PA1-053	WB, IF, FACS
Anti- OBR	Sigma, HPA030899	IF, FACS
Anti- pFAK	Abcam, ab39967	IF
Anti- FAK	Santa Cruz, sc557	WB, IF
Anti- GAPDH	Santa Cruz, sc25778	WB
Anti- Sca-1	Abcam, ab25031	IF
Anti- SMA	Sigma, A5228	IF, IHC
Anti- CD31	Abcam, ab30349	IF
Anti- leptin	Invitrogen, Pa1-051	IF, WB
Anti- F4/80	Abcam, ab6640	IF
Anti- CD68	Santa Cruz, sc-9139	IF
Anti- VE-cadherin	Santa Cruz, sc-6458	IF
Anti- CD117(ckit)-PE	eBioscience, 12-1171-82	FACS

Anti- CD146-FITC	eBioscience, 11-1469-41	FACS
Anti- Flk1-PE	BD Pharmingen, 555308	FACS
Anti- NG2	Abcam, ab129051	IF, FACS

2.1.3 Experimental Mice

All animal procedures were approved by the UK Home Office (PPL70/8944). C57BL/6J (wild-type) mice were purchased from Harlan, Blackthorn, Bicester, UK. RFP mice (Stock No: 005884) and leptin receptor deficient mice ($Lepr^{+/-}$, Stock No: 00697) were purchased from the Jackson Laboratory (Bar Harbour, Maine, USA). For $Lepr^{-/-}$ mice, A G to T transversion in the allele causes abnormal splicing and a 106 nt insertion in the transcript, leading to the premature termination of OBRb, which diminishes its signaling transducing function. Male mice aged 10-12 weeks were selected for surgery. All the mice were kept 2 to 5 per cage. All mice were kept on a chow diet with autoclaved food and water in a pathogen-free and temperature-controlled environment at 25°C with a 12-hour light and 12-hour dark cycle in the biological service unit (BSU) at the James Black Centre of King's College London. The $Lepr^{+/-}$ mice were paired and offspring genotypes were identified by standard PCR.

2.2 Methods

2.2.1 Mouse Adventitial Progenitor Cell Culture

The procedure used for adventitial progenitor cell culture was similar to that described previously (Y. Hu et al., 2004). In brief, the aortic arch and root, as well as part of the heart from $Lepr^{+/+}$ or $Lepr^{-/-}$ mice, were harvested under sterile conditions. Adventitial tissues were carefully collected under a dissection microscope by removing the aortic media, intima and heart tissue. The adventitial tissues were then cut into pieces and seeded onto a gelatin-coated T25 flask. The flask was incubated at 37℃, 5% CO2 upside down for 2 to 3 hours. After the attachment of adventitial tissues onto the flask, complete stem cell medium (Dulbecco's Modified Eagle's Medium (DMEM ATCC, 30-2002)) with leukemia inhibitory factor (10 ng/mL, Merck Millipore, LIF1050), 2-mercaptoethanol (0.1 mM, GIBCO), 100U/mL penicillin (GIBCO), 100U/mL streptomycin (GIBCO) and 2 mM L-glutamine (GIBCO) was added for 5 days. When the cells reached 90% confluency, they were washed with PBS and trypsinized. The cells were passaged at a ratio of 1:4 every two or three days. The medium was changed every 2 days.

2.2.2 Sca-1$^+$ Progenitor Cell Isolation and Culture

When reached 90% confluency, the Sca-1$^+$ adventitial progenitor cells were isolated using the Sca-1$^+$ microbeads kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instruction. Briefly, Sca-1$^+$ cells were trypsinized and the cell number was then determined by cytometer. The cells were centrifuged and resuspended in 90 μL of buffer together with 10 μL of anti-Sca-1-FITC per 10^7 total cells. After 10-minute incubation in the refrigerator, the cells were washed, centrifuged and resuspended in 80 μL of the buffer with 20 μL anti-FITC microbeads per 10^7 total cells for 15 minutes. After washing, the cells were suspended in 500 μL of buffer and were selected using a magnetic cell separator (MACS, Miltenyi Biotec). The

column was rinsed with 500 µL of cold buffer, followed by the 500 µL cell suspension. Unlabeled cells would pass through the magnetic field, whereas Sca-1^{+} progenitor cells would stay in the column. Finally, the positive cells were collected by firmly applying the plunger supplied with the column. The Sca-1^{+} progenitor cells were cultured on 2% gelatin-coated flasks in complete stem cell medium (Dulbecco's Modified Eagle's Medium (DMEM ATCC, 30-2002)) leukemia inhibitory factor (10 ng/mL, Merck Millipore, LIF1050), 2-mercaptoethanol (0.1 mM, GIBCO), 100 U/mL penicillin (GIBCO), 100 U/mL streptomycin (GIBCO) and 2 mM L-glutamine (GIBCO) for both *in vitro* and *in vivo* studies.

2.2.3 Mouse Vascular Smooth Muscle Cell Culture

The procedure used for vascular smooth muscle cells' culture was similar to that described previously (Y. Hu et al., 2004). In brief, the aortic arch and root, as well as part of the heart from Lepr$^{+/+}$ or Lepr$^{-/-}$ mice, were harvested under sterile conditions. Medial tissues were carefully collected under a dissection microscope by removing the aortic adventitia, intima and heart tissue. The media was then cut into pieces and seeded onto a 0.04% gelatin-coated T25 flask. The flask was incubated at 37℃, 5% CO2 upside down for 2 to 3 hours. After the attachment of medial tissues onto the flask, complete medium (Invitrogen 11960085, Dulbecco's Modified Eagle's Medium, high glucose) with 10% fetal bovine serum (GIBICO 10270), 100 U/mL penicillin (GIBCO), 100U/mL streptomycin (GIBCO) and 2 mM L-glutamine (GIBCO) was added for 5 days. When the cells reached 90% confluency, they were washed with PBS and trypsinized. The cells were passaged at a ratio of 1:3 every two or three days. Medium was changed every 2 days.

2.2.4 Transwell Assay

Cell migration assessments were performed using transwell inserts with 8.0

μm micron pore membrane filters in a 24-well plate (#3422, Corning Life Science, USA) as established previously (M. M. Wong et al., 2013). Sca-1$^+$ progenitor cells were trypsinized and transferred into the upper chamber at 10^5 cells/mL in serum-free media with or without inhibitors. Meanwhile, the lower chamber was loaded with 800 μL of serum-free media with treatment (Figure 2.1). After 16-hour incubation, the upper side of the filters was carefully washed using a cotton bud to remove any non-migratory cells. Migratory cells on the underside of the filters were fixed with 4% PFA for 15 minutes, followed by a 15-minutes staining with 1% crystal violet at room temperature. Data shows average cell numbers of migratory progenitor cells in 5 random fields at 20x magnification (Figure 2.2). For the experiments involving inhibitors or antagonists, Sca-1$^+$ progenitor cells were cultured with the respective chemicals in the upper chamber only.

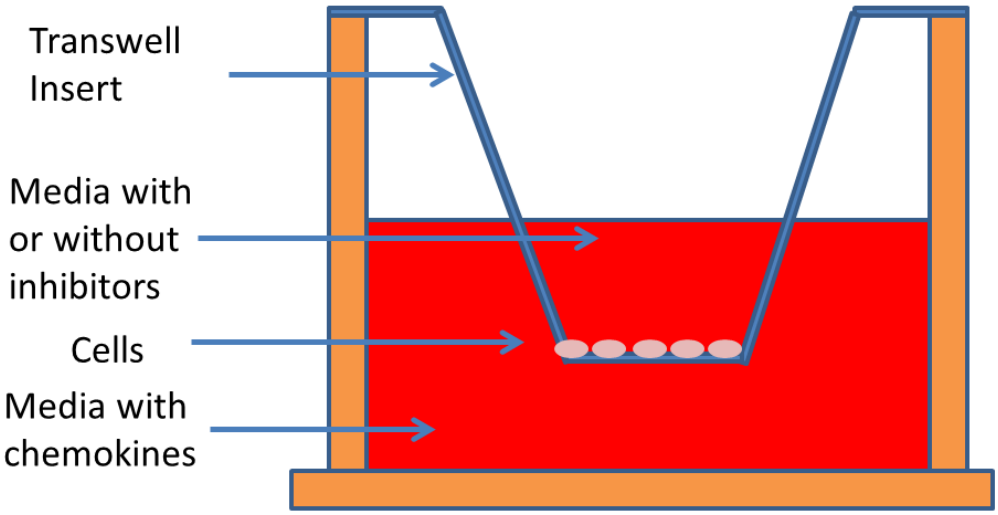


Figure 2.1. Schematic diagram of Transwell assay.

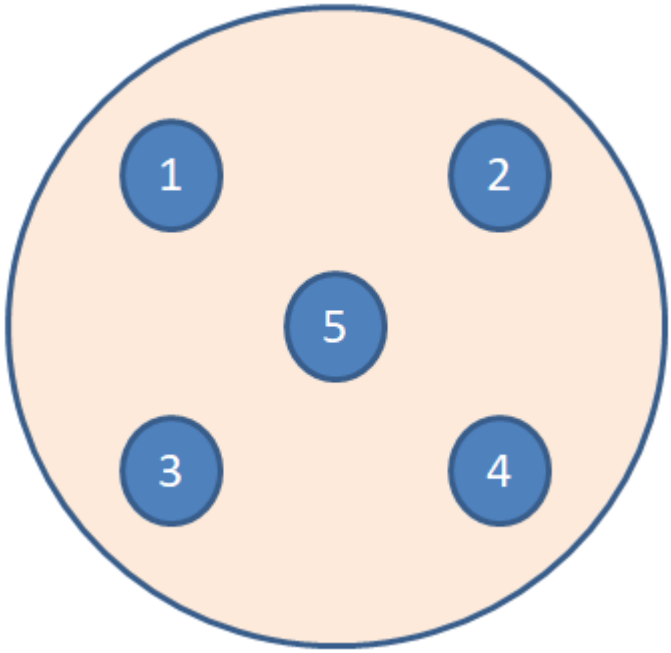


Figure 2.2 Layout of 5 random fields in transwell assay for quantification of migrated cells towards chemokines.

2.2.5 Scratch-Wound Assay

$1x10^4$ cells per well of Sca-1^+ progenitor cells were seeded in a 12-well plate in complete culture media. Once the cells reached 90% confluency, a scratch wound was made from top to bottom using a 1 mL pipette tip. The pipette was kept at a consistent angle and pressure during the scratch to ensure uniformity of width of the scratch. The wells were carefully washed twice using PBS to remove any cell debris caused by the “scratch” procedure. Treatment and/or inhibitors were added into wells with serum-free media. After 16-hour incubation, cells were fixed with 4% PFA and stained with 1% crystal violet for 15 minutes at room temperature. The migration of Sca-1^+ progenitor cells into the “wound” area was evaluated using a phase contrast microscope. Data indicated are the mean area occupied by migratory progenitor cells in 5 random fields of view at 10x magnification.

2.2.6 RNA Extraction

Cells after overnight starvation in serum-free medium were treated with 100 ng/mL leptin for 5 min, 15 min, 30 min, 1 h and 4 h. Total RNA extraction from Sca-1$^+$ progenitor cells was performed with RNeasy Mini kit (#74106, QIAGEN Inc.) According to manufacturer's instruction, cells were washed twice with PBS, disrupted by proportional amount of RLT lysis buffer and then scraped off from the 6-well plate. The lysate was transferred into a mini QIAshredder spin column and centrifuged at full speed for 2 minutes. The same volume of 70% ethanol was added to the lysate and the mixture was transferred to the RNeasy mini column for a 30-second centrifuge at full speed. The flow through was discarded and 700 μL of RW1 was added to RNeasy mini column. After 30-second centrifuge, the flow through was discarded and 500 μL of RPE was added to the column twice for washing away the ethanol. The flow through was then discarded and the column was centrifuged within a new collection tube for 2 minutes at full speed to ensure no solution outside the column. At last, the RNeasy mini column was transferred into a new 1.5 mL RNA-free tube and 40 μL of DEPC water (Invitrogen) was added to the membrane of the column, followed by 1-minute centrifuge at full speed. The RNA concentration was measured by a Nanodrop spectrophotometer ND-1000 (Thermo Scientific, UK) at λ=280nm.

2.2.7 Reverse Transcription (RT)

Reverse transcription was achieved by QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer's instructions (Table 2.3). Briefly, 1 μg of RNA template, 2 μL of gDNA wipeout buffer and enough volume of RNase-free water were mixed in the tube with a total volume of 14 μL. The tube was placed in a RT-PCR machine (TECHNE TC-412, Bibby Scientific, UK) at 42℃ for 2 minutes, after which 1 μL of RT enzyme, 1 μL of primer mix and 4 μL of RT buffer, making up to 6 μL of reaction volume, was added to the tube mentioned above. Then the mixture was incubated at 42℃ for 15

minutes and subsequently at 95℃ for 3 minutes (Table 2.4). The cDNA obtained was diluted into 100 µL by using DEPC-treated water, acquiring a final concentration of 10 ng/µL.

Table 2.3 Reverse transcription reaction mix

Reaction Component		Volume (µL)
Mix A	RNA template	x
	gDNA wipeout	2
	RNase-free water	12-x
Mix B	RT buffer	4
	RT enzyme	1
	Primer mix	1

Table 2.4 Reverse transcription program

Step	Temperature (℃)	Time (min)
1	42	2
2	Pause for Mix B	
3	42	15
4	95	3
5	10	-

2.2.8 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Quantitative real-time PCR was performed by applying SYBR green system (#PBOEM20.12, PCR Biosystems). The composition (Table 2.6) and program (Table 2.7) are listed below. The target gene was amplified in a duplex in 20 µL of PCR mixtures (10 µL of Sybr Green, 2 µL of cDNA template, 1.6 µL of optimized primers and 6.4 µL of DEPC water) which was loaded into a 96-well

plate (Eppendorf White, Eppendorf, UK). The plate was centrifuged at 1000 rpm for 5 minutes before running the program in qPCR machine. Ct values were established using EPPENDORF Mastercycler ep realplex. GAPDH served as an endogenous control. Sequences of primer sets used in this experiment are listed in Table 2.5.

Primers were designed by using DNA Integrated Technologies (IDT). (http://eu.idtdna.com/scitools/Applications/RealTimePCR/)

Table 2.5 Primers used in qPCR

Name	Sequence	NCBI reference
LEPR	5'>AGCAACTGCATCTCCAGTAATC<3'	NM_146146.2
	5'>GTGGGTTGCTGGTCTGATAAA<3'	
SM22	5'>GATATGGCAGCAGTGCAGAG<3'	NM_011526.5
	5'>AGTTGGCTGTCTGTGAAGTC<3';	
Calponin	5'>GGTCCTGCCTACGGCTTGTC<3'	XM_011242388.2
	5'>TCGCAAAGAATGATCCCGTC<3'	
ADIPOR1	5'>TGTACCCACCATGCACTTTAC<3'	NM_028320.4
	5'>CATCACAGCCATGAGGAAGAA<3'	
ADIPOR2	5'>GTATCCCTGAGCGCTTCTTT <3'	NM_197985.3
	5'> GACTCCGTGGAAGTGAACAA <3'	
CCR1	5'> CATTGTCCATGCTGTGTTTGCCCT <3'	NM_009912.4
	5'>TCTTCCACTGCTTCAGGCTCTTGT <3'	
CCR2	5'> ATCACCATTACACCTGTGGCCCTT <3'	XM_011243064.2
	5'> AGCCCTGTGCCTCTTCTTCTCATT <3'	
CCR7	5'> TCATTGCCGTGGTGGTAGTCTTCA <3'	NM_001301713.1
	5'> ATGTTGAGCTGCTTGCTGGTTTCG <3'	
CCR9	5'> TTGTGTTCATTGTGGGCACCTTGG <3'	NM_001166625.1

	5'> CATCCATTGACCAGCAGCAGCAAT<3'	
CXCR3	5'>TGTAGCCCTCACCTGCATAGTTGT<3'	NM_009910.3
	5'>GTTGTACTGGCAATGGGTGGCATT <3'	
CXCR4	5'>AGCTAAGGAGCATGACGGACAAGT<3'	XM_006529113.3
	5'>AACGCTGCTGTAGAGGTTGACAGT<3'	
CXCR5	5'>AAGCGGAAACTAGAGCCTGGTTCA<3'	NM_007551.2
	5'>ACCATCCCATCACAAGCATCGGTA<3'	
GAPDH	5'>CATGTTCGTCATGGGTGTGAACCA <3'	NM_001289726.1
	5'>ATGGCATGGACTGTGGTCATGAGT<3'	

Table 2.6 qPCR reaction mix

	Component	Volume (μL)
Master Mix	2x SYBR green	10
	10 μM primers	1.6
	DEPC-water	6.4
	10ng/μL cDNA	2

Table 2.7 qPCR reaction program

Step	Temperature (℃)	Time (min:sec)	Note
1	95	10:00	
2	95	00:15	Repeat step 2 to 3 for 40 cycles
3	60	01:00	
4	95	00:15	Build up the melting curve for the primers respectively
5	60	00:15	
6	-	20:00	
7	95	00:15	

2.2.9 Conventional Polymerase Chain Reaction (PCR)

Conventional polymerase chain reaction was performed in this project for the

identification of specific mice, such as RFP mouse and Lepr$^{-/-}$ mouse. 50 ng of cDNA was amplified to the corresponding DNA by applying Taq DNA polymerase (#1034253, Invitrogen). The PCR components (Table 2.9) and program (Table 2.10) are showed below. The products were transferred into 2% agarose gel and observed by the Biospectrum AC Imaging system and Vision worksLS software. Sequences of all primers for the identification of mice were provided by Jackson laboratory, USA. The sequences of the primers are listed in Table 2.8.

Table 2.8 primers used in conventional PCR

RFP	5'> CCCCGTAATGCAGAAGAAGA<3'	NC_000072.6
	5'> CTTGGCCATGTAGGTGGTCT <3'	
db/db	5'> AGAACGGACACTCTTTGAAGTCTC<3'	XM_017320002.1
	5'> CATTCAAACCATAGTTTAGGTTTGTGT<3'	

Table 2.9 PCR reaction mix

Components	Volume(μL)
10x PCR buffer	1.20
50mM $MgCl_2$	0.96
25mM dNTPs	0.96
20 μM Forward Primer	0.6
20 μM Reverse Primer	0.6
5 U/μL Taq DNA Polymerase	0.06
RNase-free H_2O	5.62
10 ng/μL cDNA	2

Table 2.10 PCR reaction program

Step	Temperature (℃)	Time (min:sec)	
1	94	01:30	

2	94	00:30	repeat steps 2-4 for 35 cycles
3	52	00:45	
4	72	00:45	
5	72	02:00	
6	10		hold

2.2.10 Agarose Gel Preparation and Observation

2 g of agarose powder (#16500500, Invitrogen) was added in 100 mL 1x TAE buffer (#EC872, National Diagnostic). The mixture was heated to boiling in a microwave for two minutes. Once the agarose cools down to around 60℃, 10 µL of SafeView nucleic acid stain (#NBSSV, NBS Biologicals) was added into the solution. The gel mixture was gently poured into a Horizon ® horizontal gel cast electrophoresis apparatus with the well comb inside. Once the gel reached room temperature and solidified, the gel was gently placed in the 1x TAE buffer and the comb was carefully removed. 25 µL of amplified DNA samples together with 5 µL of 6x loading buffer were loaded into each well. Electrophoresis was performed at 160 V for 20 minutes. DNA fragments were visualized under UV light by using Biospectrum Imaging System 500.

2.2.11 Phenotyping of Cultured Sca-1$^+$ Progenitor Cells and Smooth Muscle Cells

Cells were isolated from the adventitial of Lepr$^{+/+}$ and Lepr$^{-/-}$ (db/db) mice, and kept growing for few passages. The heterogeneous Sca-1$^+$ adventitial cells were purified by applying anti-Sca-1 microbeads. Flow cytometry was performed within 3 passages after sorting. Cultured lepr$^{+/+}$ and Lepr$^{-/-}$ Sca-1$^+$ progenitors were harvested by using scraptase (GenDEPOT, CA110-010). Cells were then centrifuged and resuspended in cold PBS, followed by a 30-minute staining on ice with following antibodies: Anti-Sca-1-FITC (clone

D7, abcam, ab25031), Anti-CD45-APC-Cy7 (clone 30-F11, biolegend, 103115), Anti-CD29-PE (clone HMβ1-1, BD Pharmingen, 562801), Anti-CD11b-BV785 (clone M1/70, biolegend, 101243), Anti-CD31-BV510 (clone MEC13.3, BD Horizon, 563089), Anti-CD140a-APC (clone APA5, eBioscience, 17-1401-81). Cells were analyzed with BD Accuri C6 or BD LSR Fortessa II (Becton Dickinson) flow cytometers.

2.2.12 Protein Extraction

Femoral artery or Sca-1$^+$ progenitor cells with or without treatment were lysed using RIPA buffer (#89901, Life Tech) with phosphatase inhibitor tablets (#04906845001, Roche) and protease inhibitors (#11873580001, Roche). The lysate was sonicated using a Branson Sonifier 150 at level 1 for 8 seconds twice before 45-minute incubation on ice. The lysate was then centrifuged at 15000 g for 10 minutes at 4 ºC. The supernatant was collected and transferred to a new 1.5 mL tube. The concentration of proteins was measured by performing Biorad Protein Assay (#5000006, BIO-RAD). 2 µL of the protein lysate was added to 998 µL of the 1x Bio-rad reagent. After 10-minute incubation at room temperature, the protein concentration of mixture was evaluated by using the Bio-rad spectrophotometer 3000. 2 µL of RIPA buffer was used as a blank.

2.2.13 Western Blotting Analysis

20 µg of lysate mixed with 5xSDS loading buffer was heated at 95℃ for 15 minutes before loaded into a NuPage 4-12% Bis Tris-gel (#NP0335BOX, Novex) immersed in NuPage MOPS SDS running buffer, followed by standard Western blotting procedures. Briefly, Electrophoresis was performed at 160V for 1.5 hours. The gel was then transferred onto a nitrocellulose membrane in XCell^{TM} Blot module (Invitrogen) at 35 V for 60 minutes. The membrane was then blocked with 5% milk in TBS-T (0.1% Tween,

Sigma-Aldrich) for 1 hour at room temperature, followed by 1-hour incubation with primary antibodies overnight at 4 ºC. After three washes, the corresponding secondary antibody diluted in 5% milk TBS-T was added to the membrane for 60 minutes at room temperature. After three washes, the membrane was reacted with Amersham™ ECL™ (RPN2106, Invitrogen) Western Blotting Detection Reagents (GE Healthcare) for 2 minutes at room temperature. Film (Amersham, Kodak) was exposed in the dark room by applying Compact X4 developing machine (Xograph Imaging System). Primary antibodies against pJAK2 (Cell signaling, 3771S), JAK2 (Cell signaling, 3230), pSTAT3 (Cell Signaling, 9131S), STAT3 (Cell signaling, 4904), pERK 1/2 (Santa Cruz, sc-16982-R), ERK 1/2 (Cell Signaling, 4695P), pOBR (Invitrogen, PA5-64638), OBR (Invitrogen, PA1-053), pFAK (Abcam, ab39967), FAK (Santa Cruz, sc557) and GAPDH (Santa Cruz, sc25778) were used to detect the respective proteins.

2.2.14 MAPK Pathway Phosphorylation Array

The detection of phosphorylated MAPK-related protein was performed by using MAPK pathway phosphorylation array (AAHMAPK1-4, RayBiotech). The proteins of cells with or without treatment were collected by applying lysis buffer provided in the kit. The concentration of cell lysate was measured by using detection buffer provided in the kit. Array membrane was put into a well of the incubation tray and incubated in blocking buffer for 30 minutes at room temperature. Discard the blocking buffer and pipette 1mL of diluted sample into each well and incubate for 2 hours at room temperature. After two washes, added 1 mL of the well-mixed detection antibody cocktail into each well for 2 hours at room temperature. After two washes, 2 mL of 1x HRP-Anti-Rabbit IgG was added to each well for 2 hours at room temperature. After two washes, the membrane was well cleaned and incubated with 500 µL of the well-mixed detection buffer for 2 minutes at room temperature. The

membrane was analyzed by applying an X-ray film or a chemiluminescence imaging system. The proteins detected in this array were listed in Table 2.3.

Each antibody is spotted in duplicate vertically	A	B	C	D	E	F	G	H
1	POS	POS	NEG	NEG	Akt (P-S473)	CREB (P-S133)	ERK1 (P-T202/Y204) ERK2 (P-Y185/Y187)	GSK3a (P-S21)
2								
3	GSK3b (P-S9)	HSP27 (P-S82)	JNK (P-T183)	MEK (P-S217/221)	MKK3 (P-S189)	MKK6 (P-S207)	MSK2 (P-S360)	mTOR (P-S2448)
4								
5	p38 (P-T180/Y182)	P53 (P-S15)	P70S6K (P-T421/S424)	RSK1 (P-S380)	RSK2 (P-S386)	NEG	NEG	POS
6								

Figure 2.3 layout of MAPK proteins array

2.2.15 G-LISA RhoA/Rac1/Cdc42 Activation Assay

The activation of GTPase family was determined by performing G-LISA RhoA/Rac1/Cdc42 Activation Assay (Cytoskeleton, Inc. #BK135) according to the standard protocol provided by manufactory. Briefly, cells were seeded and grown to 70% confluency, followed by the stimulation with leptin from 2 to 30 minutes. Cells were collected and lysed on ice and the concentration of cell lysate was measured by using the detection solution provided in the kit. After adding buffer blank and buffer positive control, 50 µl of equalized lysate were added to duplicate wells in an incubation plate. The plate was immediately placed on an orbital shaker at 400 rpm for 15 (Rac1 and Cdc42) or 30 (RhoA) minutes in the cold room. After two washes, 200 µl of room temperature Antigen Presenting Buffer was added to each well at room temperature for 2 minutes. After three washes, 50 µl of diluted anti-RhoA/Rac1/Cdc42 primary antibody was added to each well on an orbital shaker at 400 rpm for 30 (Rac1 and Cdc42) or 45 (RhoA) minutes at room temperature, followed by the incubation of 50 µl of diluted secondary antibody to each well for 30 (Rac1

and Cdc42) or 45 (RhoA) minutes at room temperature. After three washes, 50 µl of the mixed HRP detection reagent was added into each well at 37°C for 10 to 15 min, the reaction of which was stopped by administration of 50 µl of HRP Stop Buffer. The absorbance was measured at 490 nm by using a microplate spectrophotometer. Buffer blank was designated as assay blank.

2.2.16 Mouse Leptin ELISA

The concentration of serum leptin was measured by applying Mouse/Rat Leptin Quantikine ELISA Kit (R&D systems, # MOB00) according to the standard protocol provided by manufactory. Briefly, blood from wild-type and db/db mice was collected and allowed to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000g. Serum was collected and diluted 20 times for assays. ELISA microplate strips was firstly added 50 µL of Assay Diluent RD1W for each well. 50 µL of standard, control, or samples were then added to each well and incubated for 2 hours at room temperature. After five washes, 100 µL of Mouse/Rat Leptin Conjugate was added to each well for 2-hour incubation at room temperature. Repeat the washing procedure and add 100 µL of Substrate Solution to each well for 30 minutes at room temperature away from the light. 100 µL of Stop Solution was finally added to each well and the absorbance was determined by using a microplate reader set to 450 nm within 30 minutes. The absorbance at 540 nm or 570 nm was used as wavelength correction.

2.2.17 Immunofluorescence Staining and *En Face* Preparation

Frozen sections were fixed with 4ºC acetone and permeabilized with 0.1% Trixton X-100 in PBS for 30 minutes at room temperature. For *En Face* staining, tissues were fixed with 4% paraformaldehyde for 15 minutes and then permeabilized with 0.5% Trixton X-100 in PBS for 30 minutes. For cell staining in chamber slides (BD Biosciences), the cells were fixed with 4%

paraformaldehyde for 15 minutes and then permeabilized with 0.1% Trixton X-100 in PBS for 30 minutes. Tissues or cells were blocked in 5% swine serum for 45 minutes at room temperature prior to their incubation with primary antibodies for either 1 hour at 37 ºC or overnight at 4 ºC. After three washes with PBS, secondary antibodies were administered for 45 minutes at 37 ºC, followed by DAPI (1:5000 in PBS) staining for 5 minutes at room temperature. Frozen section or cells in chamber slides were mounted with fluorescence mounting medium (Dako). For *En Face* preparation, after the staining with DAPI the vessel was cut open along the long axis with the lumen facing up (Zou et al., 2000). The opened vessels were then transferred onto a clean slide and mounted with fluorescence mounting media (Vectashield). A confocal microscope (Leica SP5) and AxioVision Digital Imaging System (Carl Zeiss Ltd) were used for image acquisition. Primary antibodies applied in the present study were Sca-1 (Abcam, ab25031), αSMA (Sigma, A5228), CD31 (Abcam, ab30349), leptin (Invitrogen, Pa1-051), F4/80 (Abcam, ab6640), CD68 (Santa Cruz, sc-9139), VE-cadherin (Santa Cruz, sc-6458) and RFP (Abcam, ab62341). Corresponding fluorescent-conjugated IgG antibodies were used as secondary antibodies. Slides stained with secondary antibodies only served as a binding control.

2.2.18 Histological Analysis

Femoral artery was fixed with 4 % formalin overnight at 4°C prior to a machine-based dehydration. The dehydrated samples were embedded in paraffin and subsequently cut into 5 µm sections. H&E staining was performed using a standard protocol with Hematoxylin and Eosin for morphology analysis.

2.2.19 Cell Proliferation ELISA, BrdU

BrdU assay was performed using cell proliferation ELISA kit (Roche,

11669915001) according to a standard protocol provided by manufactory. Briefly, cells were cultured with leptin for different time points and then labelled with BrdU for 2 hours in a humidified atmosphere at 37°C. Incubation with Fixdenat solution and anti-BrdU POD were subsequently performed. After three washes, substrate reaction was added to each well, and the absorbance was measured at 450 nm with microplate reader.

2.2.20 RFP Labelling Cells

Lentiviral particles were generated by transfecting HEK293T cells with LV H2b_RFP plasmid (Addgene, 26001) and used to label the nucleus of adventitial cells. Sca-1^{+} progenitor cells were incubated with RFP lentivirus for 10 minutes, and the waste was carefully disposed. Expression of RFP was checked using a fluorescent microscope two days after the treatment with lentivirus.

2.2.21 Phenotyping of Cultured Sca-1^{+} Progenitor Cells And Smooth Muscle Cells

Cells were isolated from the adventitial of Lepr$^{+/+}$ and Lepr$^{-/-}$ (db/db) mice, and kept growing for few passages. The heterogeneous Sca-1^{+} adventitial cells were purified by applying anti-Sca-1 microbeads. Flow cytometry was performed within 3 passages after sorting. Cultured lepr$^{+/+}$ and Lepr$^{-/-}$ Sca-1^{+} progenitors were harvested by using scraptase (GenDEPOT, CA110-010). Cells were then centrifuged and resuspended in cold PBS, followed by a 30-minute staining on ice with following antibodies: Anti-Sca-1-FITC (clone D7, abcam, ab25031), Anti-CD45-APC-Cy7 (clone 30-F11, biolegend, 103115), Anti-CD29-PE (clone HMβ1-1, BD Pharmingen, 562801), Anti-CD11b-BV785 (clone M1/70, biolegend, 101243), Anti-CD31-BV510 (clone MEC13.3, BD Horizon, 563089), Anti-CD140a-APC (clone APA5, eBioscience, 17-1401-81), CD105-PE (clone MJ7/18, BioLegend, 120407),

CD117(ckit)-PE (clone 2B8, eBioscience, 12-1171-82), CD146-FITC (clone P1H12, eBioscience, 11-1469-41), Flk1-PE (clone AVAS 12α1, BD Pharmingen, 555308), anti-NG2 antibody (rabbit polyclonal, Abcam, ab129051), anti-Lepr (rabbit polyclonal, Sigma, HPA030899). Cells stained with secondary control only were used as negative control. Cells were analyzed with BD accuri C6 or BD LSR fortessa II (Becton Dickinson) flow cytometers.

2.2.22 Van Gieson Staining

Slides were underwent deparaffin and rehydration, following the immersion in 32 mmol/L of potassium permanganate for 10 minutes. After rinsing in dH2O, the slides were immersed in 70 mmol/L of oxalicacid dehydrate for 5 minutes, washing in several changes of dH2O. The slides were again rinsed in 70% ethanol, followed by a 3-hour incubation in Miller's stain. After rinsing in 70% ethanol and dH2O, the slides were incubated in Van Gieson solution for 5 minutes. After washing in dH2O, the slides were dried and mounted with mounting medium.

2.2.23 Mouse Femoral Artery Denudation Injury and Cell Delivery

The procedure used for the mouse model is similar to that established previously (Zeng et al., 2006). Wild-type or leptin receptor deficient mice (db/db) were anesthetized with ketamine and medetomidine hydrochloride. A groin incision was made under a surgical microscope. An arteriotomy was made in the epigastric branch of femoral arteries. A 0.014' guide wire (Hi-Torque, Cross-it 200XT) was inserted into the femoral artery above the bifurcation of the abdominal aorta. The guide wire was gently pulled back and forth three times, leading to the endovascular injury of the arteries. After the removal of the guide wire, the femoral artery was ligated. Sham-injury arteries

without passage of the guide wire were used as a control. After the injury, 1×10^6 (RFP) progenitor cells with or without treatment in 30 μL of Matrigel Basement Membrane Matrix per artery were delivered to the adventitial side of injured artery. The arteries were harvested on days 7, 14 or 28 post-surgery for *En Face*, frozen section or paraffin sections. The areas of neointima, media and adventitia of the arteries were analyzed as shown in Figure 2.4.

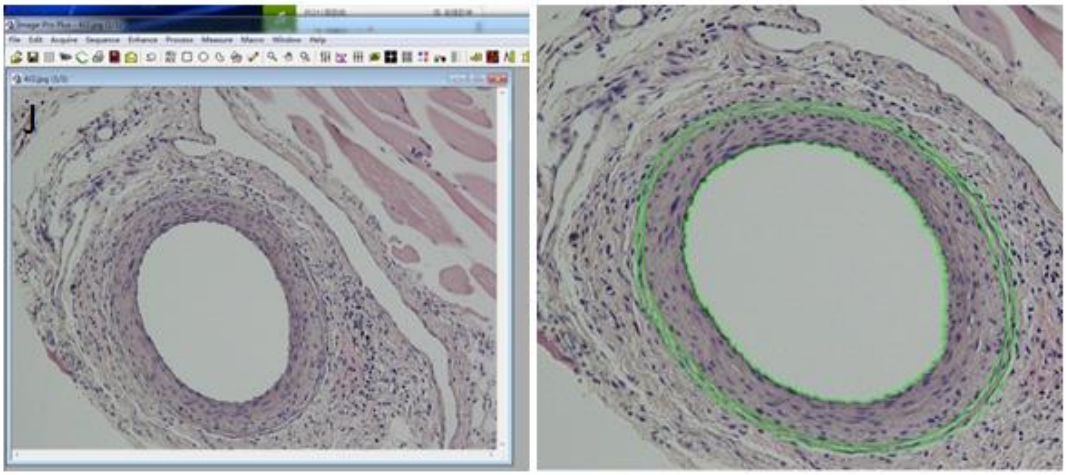


Figure 2.4 Image pro plus was applied for graph analysis. Opened the image by “image pro plus 6.0”; used irregular wand to draw the outlines of lumen (A), internal elastic lamina (B) and external elastic lamina (C); calculated the areas of three circles. Three different circles were built. B-A standed for neointima area and C-B meant media area. (Neointima area / medial area,I/M) = (B-A) / (C-B) and (medial area percentage）=（C-B) / C

2.2.24 Immunohistochemical Staining

The procedure used in present study is similar to that established previously (Zeng et al., 2006). Immunohistochemistry was achieved by using Vectastain ABC HRP kit (Vector laboratories, PK-6100). Briefly, praffin sections were incubated with αSMA (Sigma, A5228, 1:200) primary antibody for 1 hour at

room temperature after fixation and blocking. Diluted biotinylated secondary antibody was then applied for 30 minutes at room temperature. After washing, sections were incubated in peroxidase substrate solution until desired stain intensity showed up. The sections were then rinsed, counterstained, cleared and mounted.

2.2.25 Statistical Analysis

Data represented as the mean and standard error of the mean (S.E.M.) of at least three individual experiments. Data were analyzed using Graphpad Prism 6 with unpaired and two-tailed Student's t-test. For data involved in more than two groups, ANOVA test was applied followed by Dunnett's multiple comparison test. The mean of each column with different treatments was compared to the mean of the column named either "Ctr", "Control" or "0".. Significance was considered as p-value <0.05.

Chapter 3
Results

3.1 The Migration of Sca-1^{+} Adventitial Progenitor Cells Could Be Inhibited or Induced by Different Adipokines.

Obesity leads to changes in the levels of plasma adipokines such as adiponectin, leptin, visfatin, and resistin, all of which may influence cardiovascular system. To determine whether Sca-1^{+} progenitors could respond to these adipokines, migratory assays including transwell (Figure 3.1A) and wound-healing (Figure 3.1B) in response to different adipokines were performed. Our data demonstrated that 16-hour treatment of 100 ng/mL leptin and 10 ng/mL resistin could significantly induce the chemotaxis of Sca-1^{+} progenitor cells in transwell assay and migration in wound-healing assay. However, Sca-1^{+} progenitor cells displayed a reduced migration in response to 10 ng/mL adiponectin. It is well established that leptin receptor b played a crucial role in leptin-induced cell response, whereas the identity of the receptors of resistin are still under debate. Single cell transcriptome analysis of Sca-1^{+} progenitor cells (data not shown, in press) in the previous study from our lab revealed that Sca-1^{+} progenitor cells from both wild-type and apoE$^{-/-}$ mice expressed leptin receptor b (OBR). Since the migration of Sca-1^{+} progenitor cells contributes to vascular remodeling, the relationship between leptin, adventitial progenitor cells and neointimal formation drew our attention.

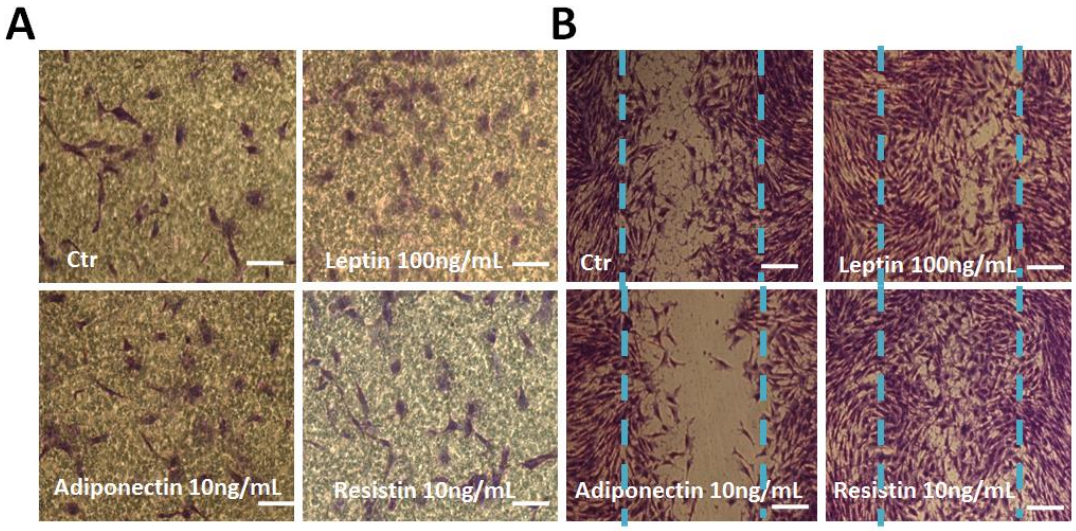


Figure 3.1. The migration of Sca-1$^+$ adventitial progenitor cells could be inhibited or induced by different adipokines. A, Chemotaxis of Sca-1$^+$ progenitor cells in response to 10 ng/mL adiponectin (n=3), 10 ng/mL resistin (n=3) and 100 ng/mL leptin (n=6) was evaluated by applying 1% crystal violet staining after 16-hour incubation (scale bars, 50 μm). **B**, Migration of Sca-1$^+$ progenitor cells in response to 10 ng/mL adiponectin (n=3), 10 ng/mL resistin (n=3) and 100 ng/mL leptin (n=6) was documented by using an 8.0 μm transwell system. Serum-free cultured medium and dimethyl sulfoxide (DMSO) without treatment was used as controls for the migration assays above.

3.2 Leptin Enhanced the Migration of Sca-1$^+$ Progenitor Cells

To investigate whether leptin was involved in the migration of Sca-1$^+$ adventitial progenitor cells, we performed an *in vitro* transwell assay. 1*10^6 Sca-1$^+$ progenitor cells after sorting were transferred into upper chamber for the incubation of different time length with 100 ng/mL leptin in the bottom chamber. Cell numbers on the underside of the transwell membrane were counted after staining with 1% crystal violet. We found that treatment with 100 ng/mL of leptin markedly induced the chemotaxis of Sca-1$^+$ progenitor cells in a time-dependent way with a peak of migratory difference after 16-hour incubation (Figure 3.2A and B). Therefore, we performed following migratory assays for an incubation of 16 hours with different concentration of leptin. Our data indicated that leptin could substantially induce the chemotaxis of Sca-1$^+$ progenitors in a dose-dependent manner (Figure 3.3A and B), with maximal chemotaxis at 100 ng/mL of leptin after 16-hour incubation. Wound healing assay also allows researchers to study cell migration. Consistent with the results in transwell assay, we confirmed that leptin could also induce the migration of Sca-1$^+$ progenitor cells in wound-scratch assays especially at high concentration of leptin (Figure 3.4A and B). Taken together, leptin could induce the migration and chemotaxis of Sca-1$^+$ progenitor cells *in vitro*.

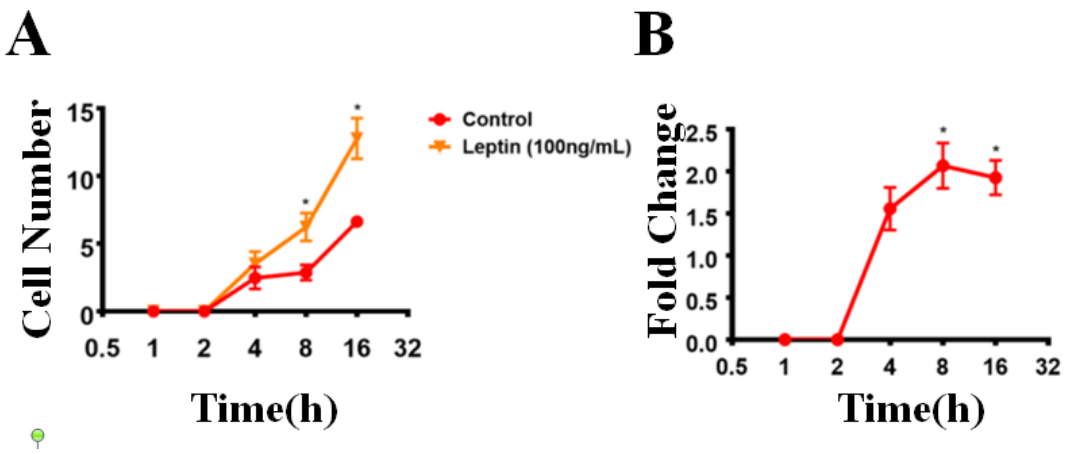


Figure 3.2. Leptin can induce the migration of Sca-1$^+$ adventitial progenitor cells in a time-dependent manner. A, Chemotaxis of Sca-1$^+$ progenitor cells at different time points with or without the treatment of 100 ng/mL recombinant leptin was documented by using an 8.0 μm transwell assay (n=4). **B**, The ratio between control and leptin-treated group was analyzed at each time point (n=4). Serum-free cultured medium without leptin treatment was used as a control for the migration assays above. Migration index for transwell assays was defined as the mean ratio of treatment to control of cell numbers counted per 5 random fields at 20X magnification. Fold change represented the ratio of the number of cells in experimental groups compared to control. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

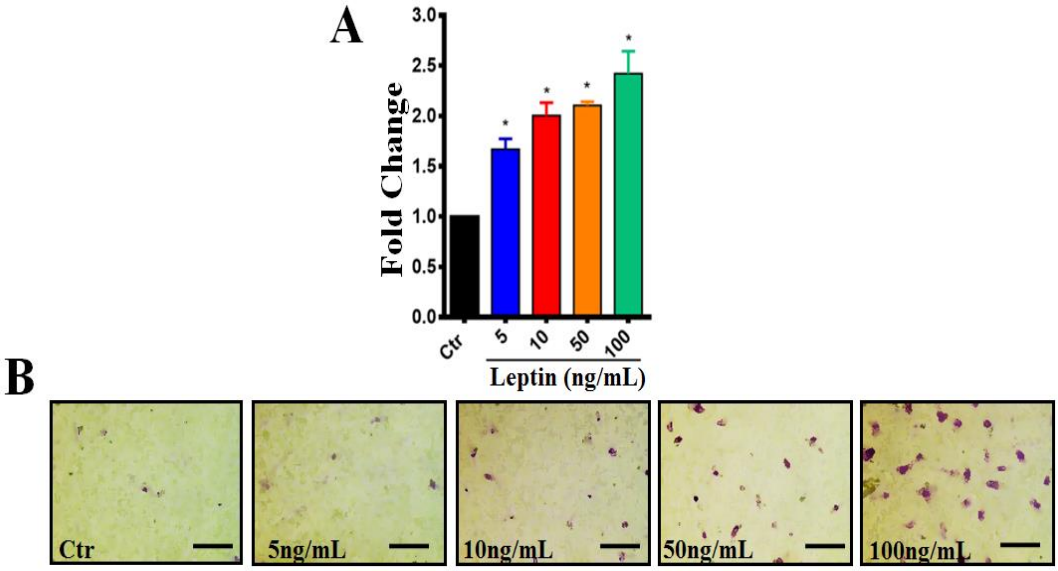


Figure 3.3. Leptin can induce the chemotaxis of Sca-1$^+$ adventitial progenitor cells in a dose-dependent manner. A and **B**, Chemotaxis of Sca-1$^+$ progenitor cells in response to an increasing gradient of leptin in an 8.0 μm transwell system was identified by applying 1% crystal violet staining after 16-hour incubation (scale bars, 50 μm, n=6). Serum-free cultured medium without leptin treatment was used as a control for the migration assays above. Migration index for transwell assays was defined as the mean ratio of treatment to control of cell numbers counted per 5 random fields at 20X magnification. Fold change represented the ratio of the number of cells in experimental groups compared to control. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

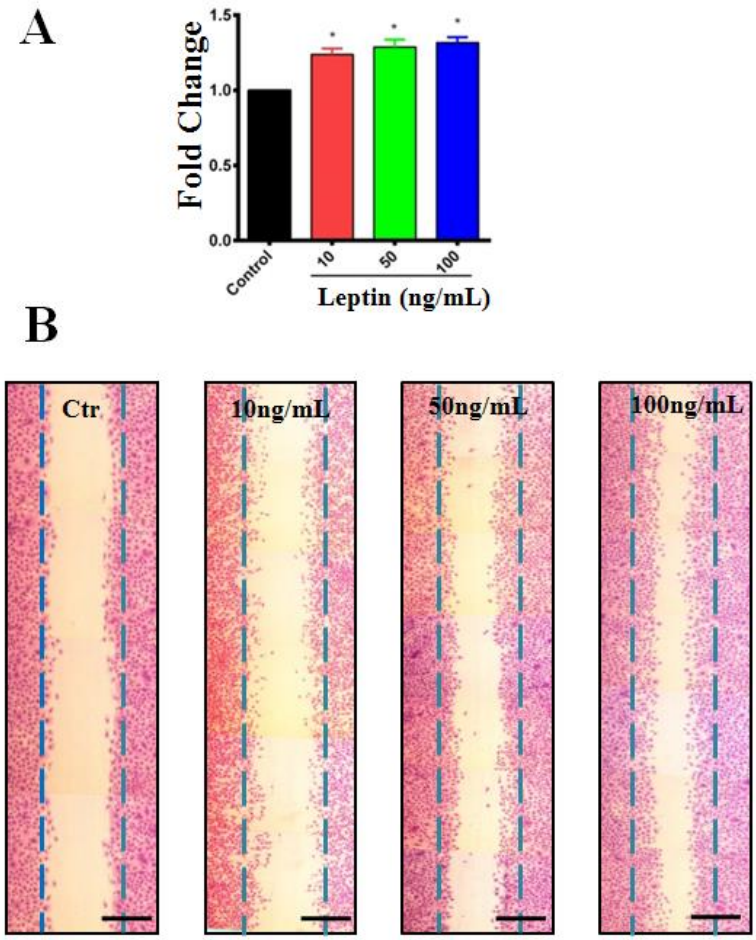


Figure 3.4. Leptin can induce the migration of Sca-1$^+$ adventitial progenitor cells in a time-dependent manner. A and **B**, Migration of Sca-1$^+$ progenitor cells in response to an increasing gradient of leptin after 16-hour incubation was evaluated in a scratch assay by following crystal violet staining (scale bars, 100 µm, n=6). Serum-free cultured medium without leptin treatment was used as a control for the migration assays above. Migration index for scratch assays was defined as the mean ratio of treatment to control of migrated area counted per 5 random fields at 10X magnification. Fold change represented the ratio of the number of cells in experimental groups compared to control. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

3.3 Leptin Did Not Enhance the Proliferation of Sca-1$^+$ Progenitor Cells

Although transwell and wound-healing assays demonstrated promising results of leptin-induced cell migration, higher numbers of cells could also be caused by cell proliferation. To assess the potential effect of leptin on cell proliferation during migratory assays, BrdU assay was performed. Data demonstrated that leptin could not enhance the proliferative ability of Sca-1$^+$ progenitor cells 16 hours after treatment (Figure 3.5A). There is no difference between the groups even in cells stimulated with high concentrations of leptin. Taken together, leptin could increase the chemotaxis and migration of Sca-1$^+$ progenitor cells *in vitro*, particularly at a high concentration of 100 ng/mL. Therefore, subsequent experiments including G-LISA, qPCR, Western blotting and immunofluorescent staining were performed with a concentration of 100 ng/mL of leptin.

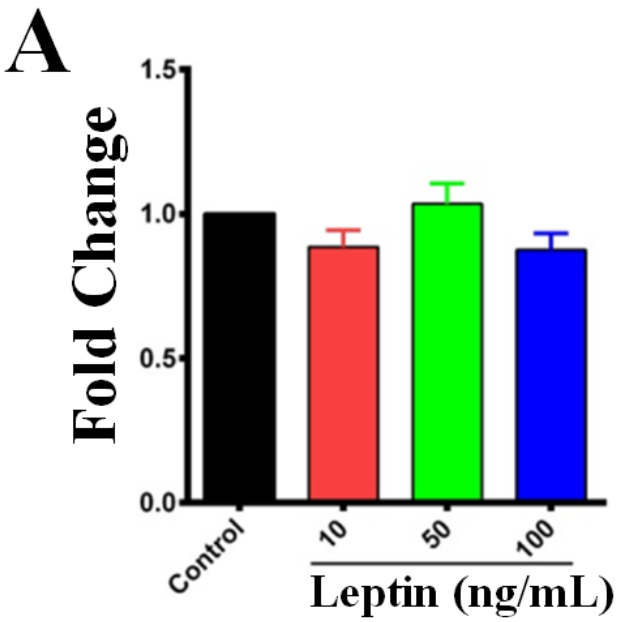


Figure 3.5. Leptin cannot induce the proliferation of Sca-1$^+$ adventitial progenitor cells. A, Proliferation of Sca-1$^+$ progenitor cells was examined by BrdU assay after 16-hour incubation with leptin (n=6). Serum-free cultured medium without leptin treatment was used as a control for the proliferation assays. All graphs are shown as mean ± SEM.

3.4 Sca-1$^+$ Progenitor Cells Expressed Leptin Receptor

Leptin receptors have six isoforms but only leptin receptor b (OBR) is considered to functionally transduce downstream signals. Since leptin could induce the migration of Sca-1$^+$ progenitor cells, the existence of OBR was analyzed in Sca-1$^+$ progenitor cells isolated from the adventitia of the aortas of C57BL/6J mice. Although we had data from single cell transcriptome analysis showing the expression of Lepr on Sca-1$^+$ progenitor cells, we still further confirmed the expression of OBR on Sca-1$^+$ progenitor cells by conventional PCR (Figure 3.6A and B) and Western blotting analysis (Figure 3.6C and D). The expression of OBR was not upregulated in response to 100 ng/mL of leptin at either RNA or protein levels. The existence of OBR$^+$ / Sca-1$^+$ adventitial cells was also identified by performing the immunostaining of aorta (Figure 3.7A) and femoral artery (Figure 3.7B) from wild-type mouse. We also performed immunostaining with CD29, CD34, CD45, Sca-1 and OBR primary antibodies on the Sca-1$^+$ progenitor cells which had migrated on the lower side after transwell assays. After the fixation with 4% PFA for 15 minutes, the membrane was cut into small pieces and pasted to a slide, followed by a standard protocol of immunofluorescence. The presence of OBR and Sca-1 (Figure 3.8A) was again confirmed. We also discovered that Sca-1$^+$ progenitor cell which had migrated in response to leptin could express CD29 (Figure 3.8B) and CD34 (Figure 3.8C) but not CD45 (Figure 3.8D). Thus, we confirmed that Sca-1$^+$ progenitor cells expressed leptin receptor as well as other progenitor markers.

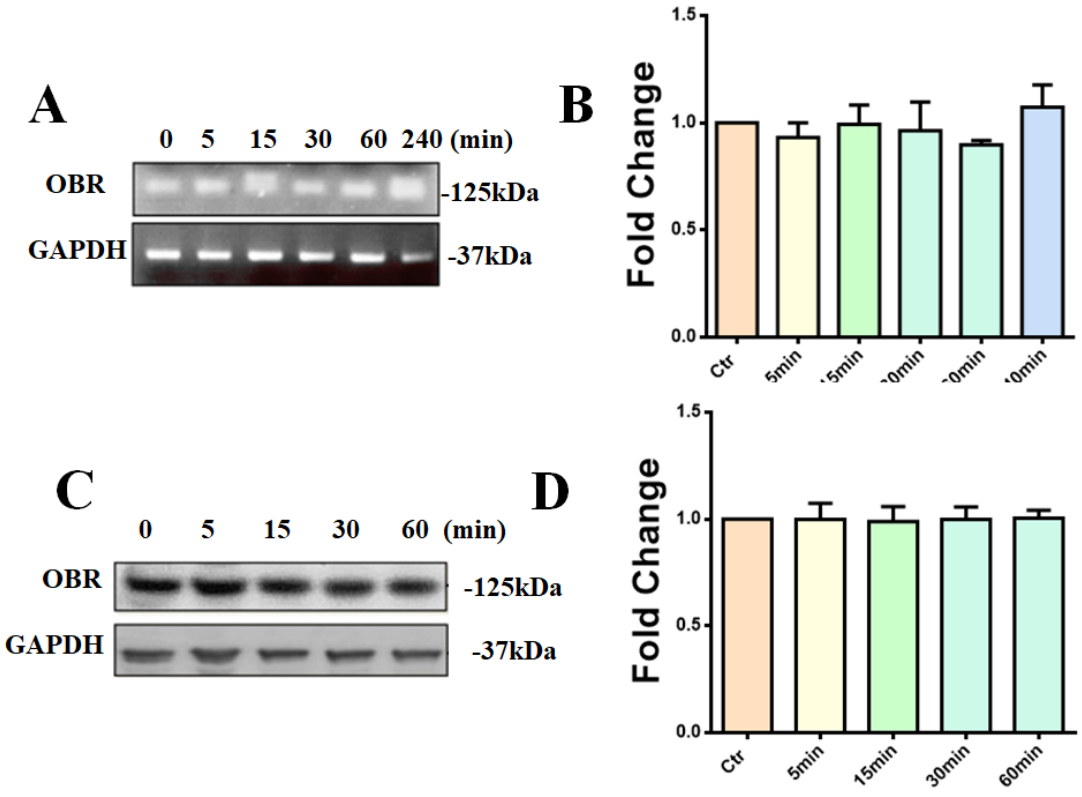


Figure 3.6. Leptin could not increase the expression of OBR on Sca-1$^+$ adventitial progenitor cells. A and **B**, The presence of leptin receptor b (OBR) in Sca-1$^+$ progenitor cells was confirmed by performing conventional PCR (n=4). **C** and **D**, The presence of leptin receptor b (OBR) in Sca-1$^+$ progenitor cells was confirmed by Western blotting (n=7). Untreated cells served as a control. Images shown are representative of at least 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001.

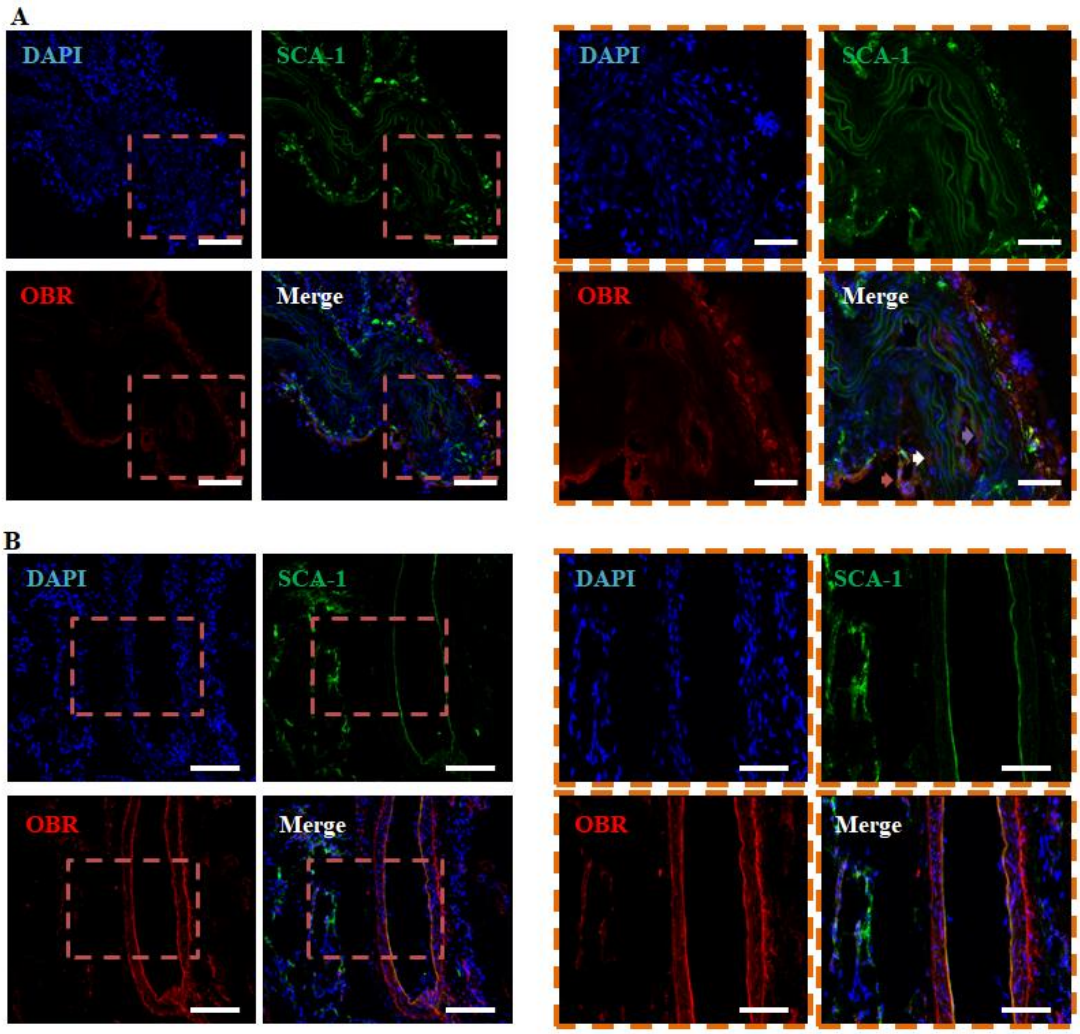


Figure 3.7. Sca-1$^+$ OBR$^+$ cells reside mainly in the adventitia of aorta and femoral artery. A, Cross sections of aorta from wild-type were analyzed by immunofluorescence for Sca-1 and OBR (scale bars, 100 and 50 μm, n=4). Red arrow indicated Sca-1$^+$ OBR$^+$ adventitial cells. White arrow indicated Sca-1$^+$ OBR$^+$ medial cells. Purple arrow indicated Sca-1$^+$ OBR+ endothelial cells. **B**, Cross sections of femoral artery from wild-type mice were analyzed by immunofluorescence for Sca-1 and OBR (scale bars, 50 and 25 μm, n=4).

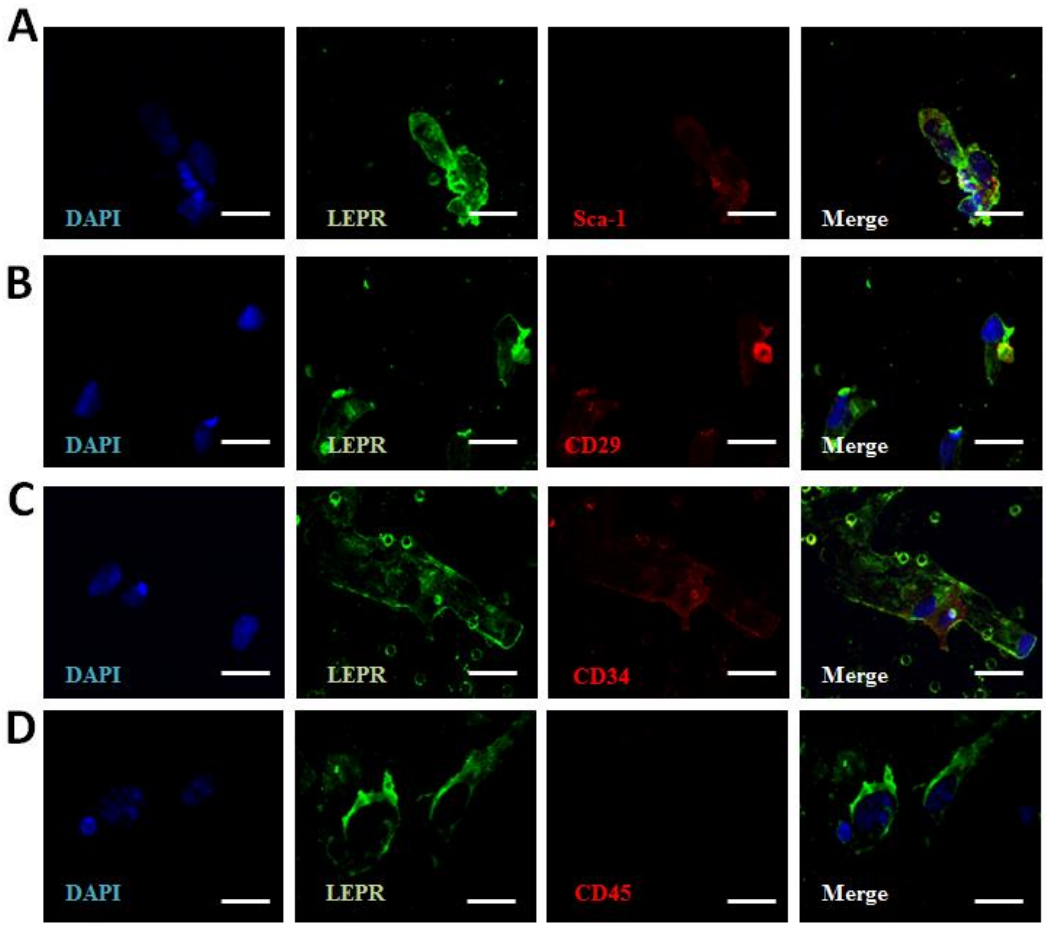


Figure 3.8. The expression of progenitor markers in leptin-induced migrated cells. Migrated cells after trans well assay were analyzed by immunofluorescence for Sca-1(**A**), CD29 (**B**), CD34(**C**), CD45(**D**), and OBR (scale bars, 50 μm, n=3). Images shown are representative of at least 3 independent experiments.

3.5 Absence of OBR Did Not Affect the Nature of Sca-1$^+$ Progenitor Cells

To further investigate the role of OBR in Sca-1$^+$ progenitor cells, the adventitia of aorta of confirmed db/db mice was harvested for the isolation of Lepr$^{-/-}$ Sca-1$^+$ progenitor cells. Conventional PCR was performed to confirm the genotype of db/db mice using the primers and protocols provided by Jackson laboratory (Figure 3.9A). Uninjured femoral arteries from either wild-type or db/db mice were harvested. Immunostaining of frozen section of femoral arteries from db/db (Figure 3.9C) and wild-type (Figure 3.9B) mice confirmed that the femoral arteries from db/db mice did not show any expression of leptin receptor in femoral artries and that Sca-1$^+$ cells showed a similar distribution in the vascular wall of db/db and wild-type mice. Data demonstrated that OBR expressed in all three layers of femoral artery in wild-type mice, whereas Sca-1 mainly expressed in the intima and adventitial in both wild-type and db/db mice. There was no difference for the expression of Sca-1$^+$ progenitor cells between wild-type and db/db mice before vessel injury (Figure 3.10A, B and C). However, a significant reduction of the expression of Sca-1$^+$ progenitor cells was observed in db/db mice post-surgery (Figure 3.10D), indicating that the migratory ability of Lepr$^{-/-}$ cells may be impeded *in vivo*. To investigate whether the absence of OBR affects the phenotype of Sca-1$^+$ progenitor cells, we performed flow cytometry for hematopoietic, mesenchymal and progenitor markers on Lepr$^{+/+}$ and Lepr$^{-/-}$ Sca-1$^+$ progenitor cells. Data revealed that there were no differences in the expression of any markers between Lepr$^{+/+}$ (Figure 3.11 and 3.12A) or Lepr$^{-/-}$ (Figure 3.11 and 3.12B) adventitial progenitor cells. Both Sca-1$^+$ progenitor cells populations were negative for hematopoietic markers CD45 and CD11b, endothelial marker CD31, excluding the possibilities that Sca-1$^+$ progenitor cells originated from endothelium or bone marrow. Moreover, data showed similar staining for CD29 (99.9%) and CD140a (99%). Collectively, our results

showed that the absence of OBR from db/db mice either did not affect Sca-1^{+} progenitor cells phenotypic characteristics.

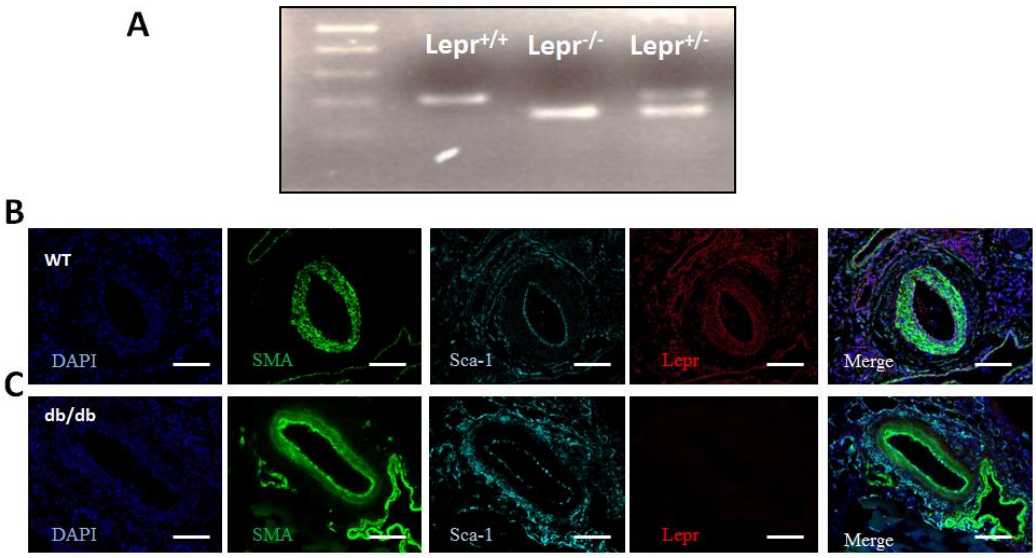


Figure 3.9. The identification of Lepr$^{-/-}$ mice was achieved by applying conventional PCR and immunostaining. A, Conventional PCR was performed for the identification of every Lepr$^{-/-}$ mice (n=29). B, Cross sections of uninjured femoral artery from wild-type were analyzed by immunofluorescence for αSMA, Sca-1 and lepr (scale bars, 50 μm, n=4). C, Cross sections of uninjured femoral artery from db/db were analyzed by immunofluorescence for αSMA, Sca-1 and Lepr (scale bars, 50 μm, n=4).

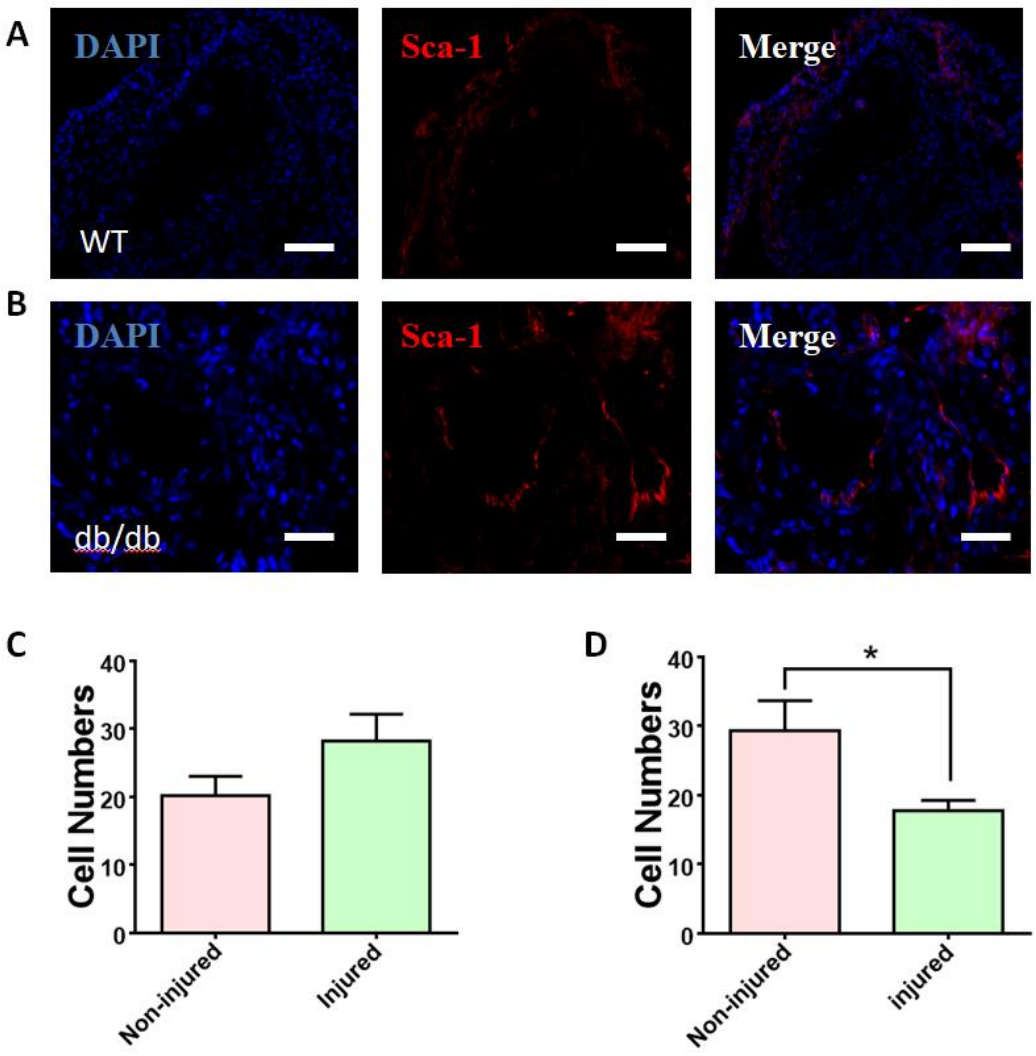


Figure 3.10. The quantification of Sca-1⁺ cells two weeks after vessel injury was analyzed in db/db and wild-type. A, Cross sections of injured femoral artery from wild-type were analyzed by immunofluorescence for Sca-1 (scale bars, 50 μm, n=4). **B**, Cross sections of injured femoral artery from db/db were analyzed by immunofluorescence for Sca-1 (scale bars, 50 μm, n=4). **C**, The semi-quantification of number of Sca-1⁺ adventitial cells was evaluated in wild-type mice before and two weeks post-surgery (n=4). **D**, The semi-quantification of number of Sca-1⁺ adventitial cells was evaluated in db/db mice before and two weeks post-surgery (n=4). All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

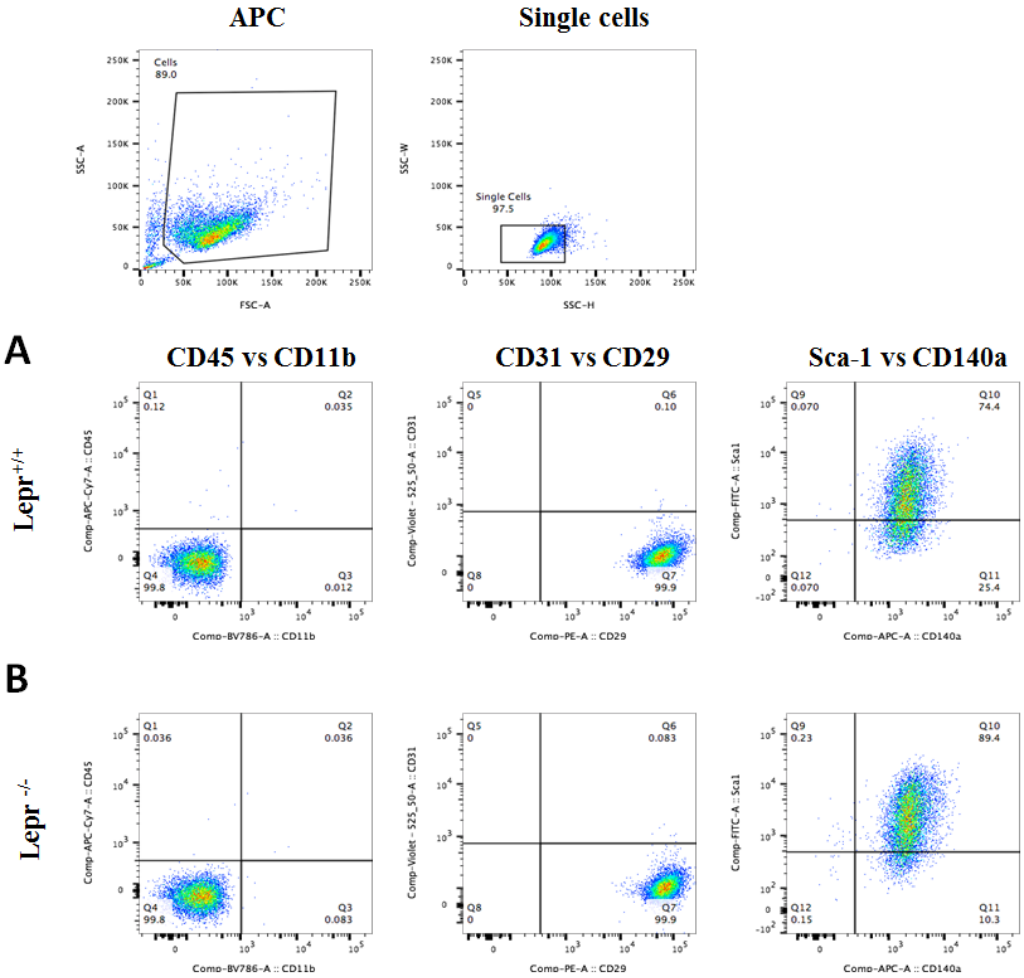


Figure 3.11. Phenotype of wild type and Lepr KO of Sca-1^{+} adventitia progenitor cells. Cells were originated from either wild-type or db/db mice. A, Phenotype of wild type Sca-1^{+} adventitia progenitor cells with primary antibodies of CD45, CD11b, CD31, CD29, CD140a and Sca-1. B, Phenotype of Lepr$^{-/-}$ Sca-1^{+} adventitia progenitor cells with primary antibodies of CD45, CD11b, CD31, CD29, CD140a and Sca-1.

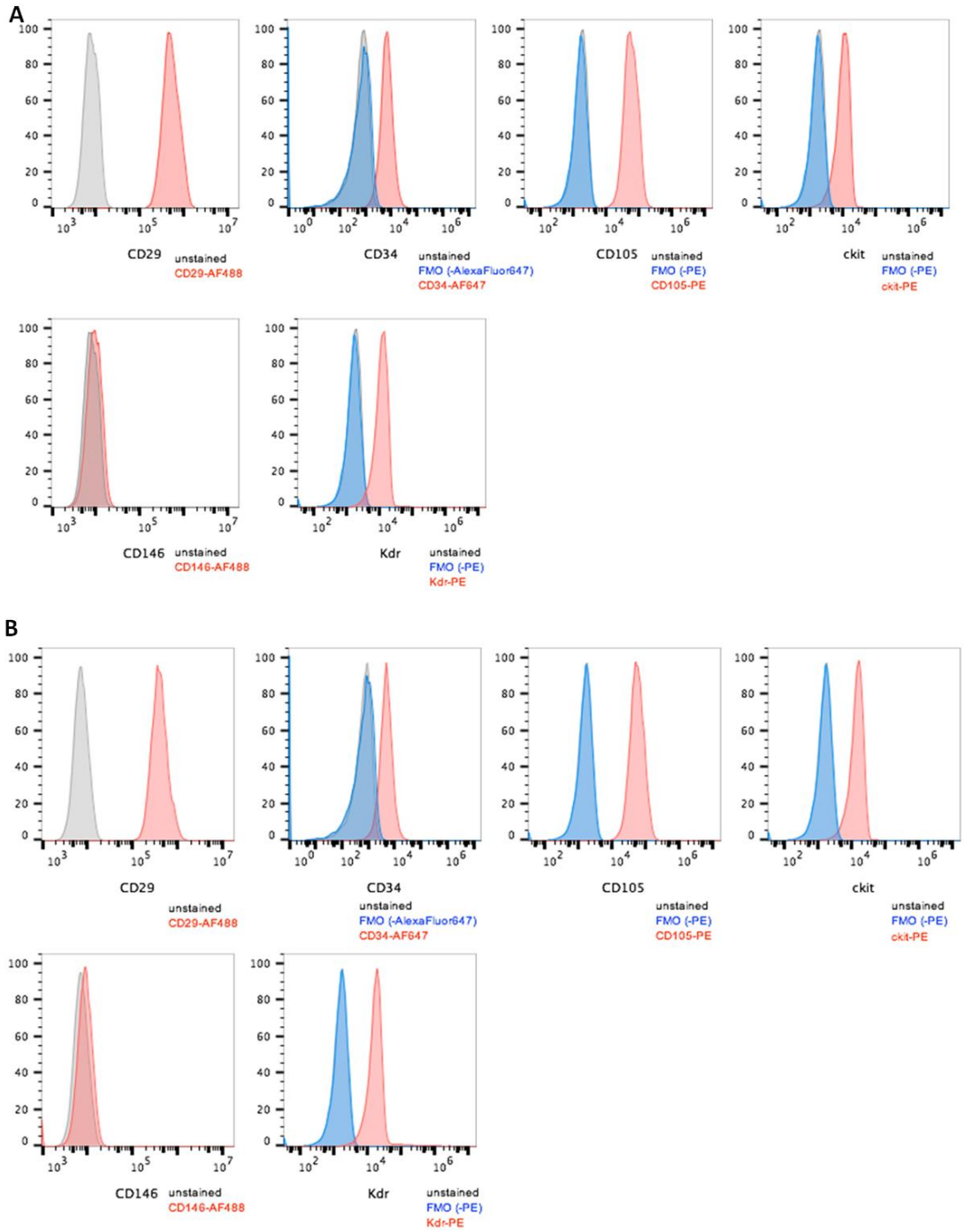


Figure 3.12. Phenotyping of Lepr$^{+/+}$ and Lepr$^{-/-}$ of Sca-1^{+} adventitia progenitor cells. A, Phenotyping of Lepr$^{+/+}$ Sca-1^{+} adventitia progenitor cells with primary antibodies of CD29, CD34, CD105, c-kit, CD146 and Kdr. **B**, Phenotyping of Lepr$^{-/-}$ Sca-1^{+} adventitia progenitor cells with primary antibodies of CD29, CD34, CD105, c-kit, CD146 and Kdr.

3.6 OBR-STAT3-MAPK Pathways and Rho GTPase Are Activated in Sca-1$^+$ Cells in Response to Leptin

To understand the underlying mechanism behind the Sca-1$^+$ progenitor cells migration in response to leptin, we next sought to elucidate OBR downstream signaling pathways. Sca-1$^+$ progenitor cells were starved overnight in serum-free medium, prior to the treatment with 100ng/mL of leptin for different time points. Cells were then collected by scrapers and protein lysis buffer for western blotting assays. The signaling pathways of leptin were well-established in other types of cells. The activation of STAT3 is known to be important steps in leptin-induced migration in other cell types, such as breast cancer cells and liver cancer cells (Dieudonne et al., 2002; Saxena et al., 2007). Treatment with 100 ng/mL of leptin for different time points demonstrated that phosphorylation STAT3 was activated from 5 minutes and reached maximal expression 15 minutes after treatment (Figure 3.13A and B).

MAPK/MEK/ERK pathway belongs to the well-defined Ras/Raf/MAPK signaling cascade, which can be activated by numerous stimuli including leptin. Previous studies showed that leptin could participate in the apoptosis of osteoblastic progenitor lineage, the migration of endothelial cells and the hypertrophy of vascular smooth muscle cells *via* ERK 1/2 signaling pathway (Zeidan et al., 2005). However, the role of this pathway in adventitial progenitor cells remained unknown. Our data showed that pERK1/2 and pMEK1/2 were activated within 5 minutes after the treatment in adventitial progenitor cells, without any change in total MEK1/2 (Figure 3.17A and C) and ERK1/2 (Figure 3.13C and D).

Rho GTPase family members have been reported as critical factors for cell migration.(Horwitz & Parsons, 1999) To elucidate whether RhoA, Cdc42 or Rac1 were involved in Sca-1$^+$ progenitor cell leptin-induced migration, G-LISA activity assay was performed which was, in comparison to pull-down assay, a

novel and efficient approach to quantify the activation of GTPase family. Treatment with 100 ng/mL of leptin led to the early activation of Cdc42 (Figure 3.14A) and Rac1 (Figure 3.14B) within 5 minutes but not of RhoA.

Vinculin is a member of cytoskeleton anchoring in F-actin protein. Vinculin is involved in linking integrin adhesion molecules to the actin cytoskeleton, associated with cell-cell and cell-matrix junctions. Phosphorylated Focal Adhesion Kinase (pFAK), also known as protein tyrosine kinase 2 (PTK2), contributes to cell adhesion and migration. Previous studies from our laboratory demonstrated that pFAK and vinculin were both relocated in Sca-1$^+$ progenitor cells after a wide range of stimuli. Our results revealed that the relocations of cytoskeleton-related proteins such as phosphorylated FAK (pFAK) (Figure 3.15) and vinculin (Figure 3.16) were observed 15 minutes after the treatment in Sca-1$^+$ progenitor cells, indicating the final stages of cell migration. Taken together, these results imply that leptin can induce the activation of pSTAT3, pMEK, pERK1/2, Cdc42, Rac1, pFAK, and vinculin.

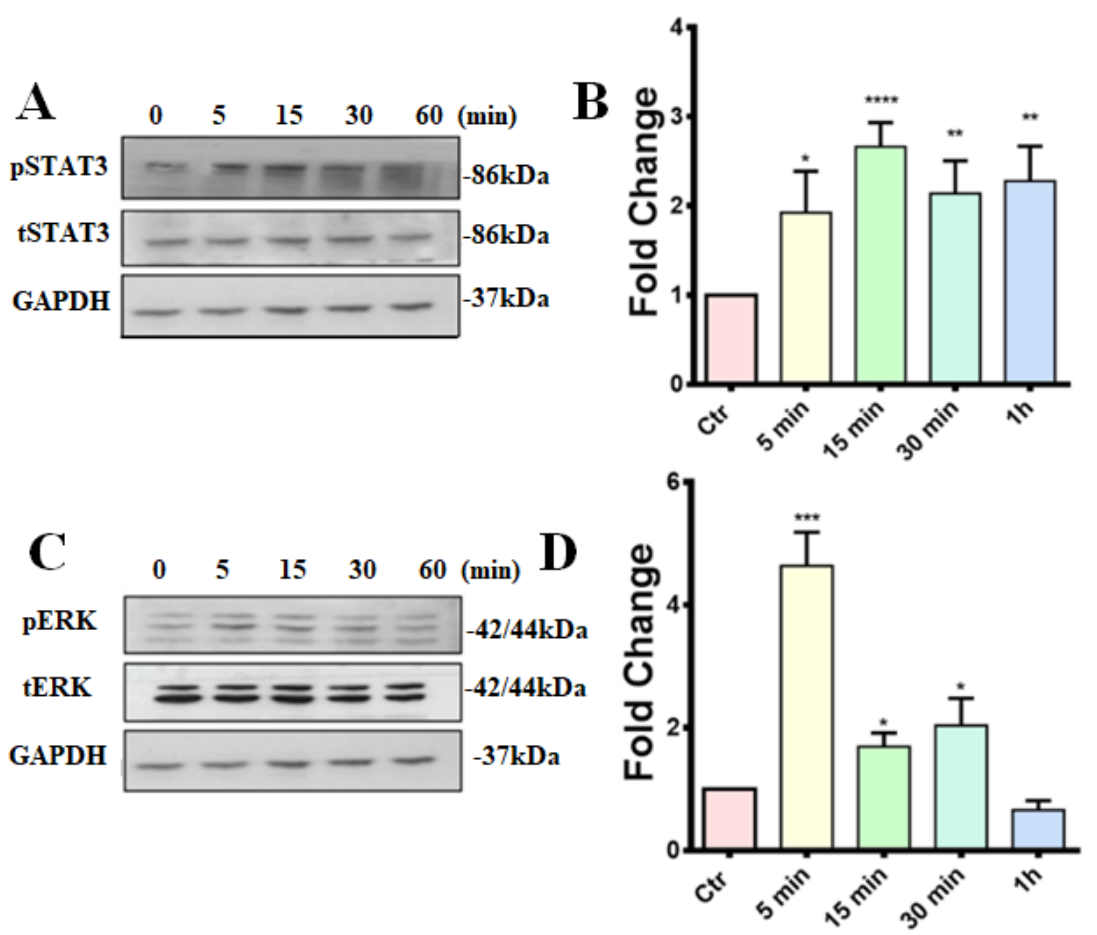


Figure 3.13. Leptin activated the pSTAT3 and pERK1/2 signaling pathways in Sca-1^{+} adventitial progenitor cells. **A** and **B**, The activation of pSTAT3 was detected by performing Western blotting on Sca-1+ progenitor cells in response to 100 ng/ml of leptin (n=6). **C** and **D**, The activation of pERK1/2 was detected by performing Western blotting on Sca-1+ progenitor cells in response to 100 ng/ml of leptin (n=10). Untreated cells served as a control. Images shown are representative of at least 3 independent experiments. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

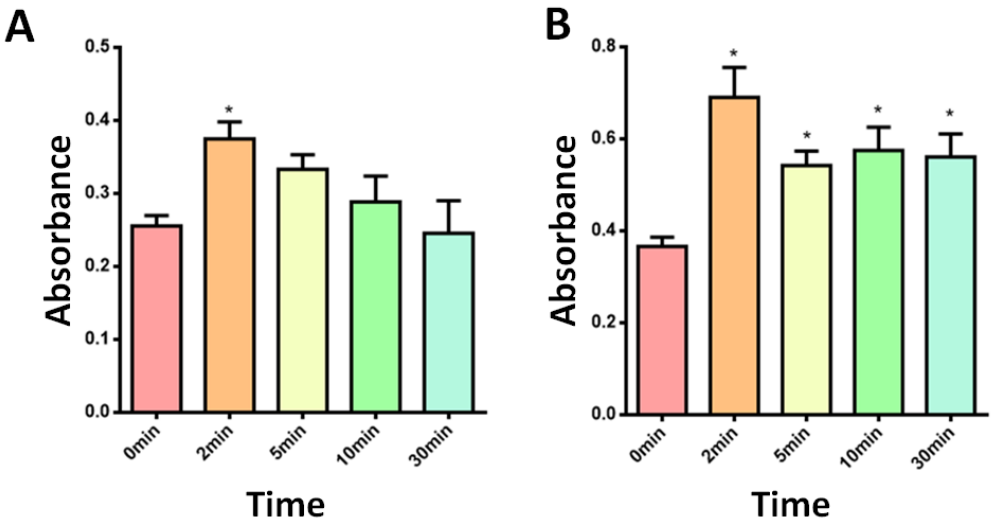


Figure 3.14. Leptin induces the migration of Sca-1^{+} adventitial progenitor cells *via* the activation of GTPase Cdc42 and Rac1. A and **B**, The quantification of activated GTPase Cdc42 (**A**) and Rac1 (**B**) was evaluated in response to 100ng/mL leptin by performing G-LISA G-protein activation assays (n=3). Untreated cells were served as control. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

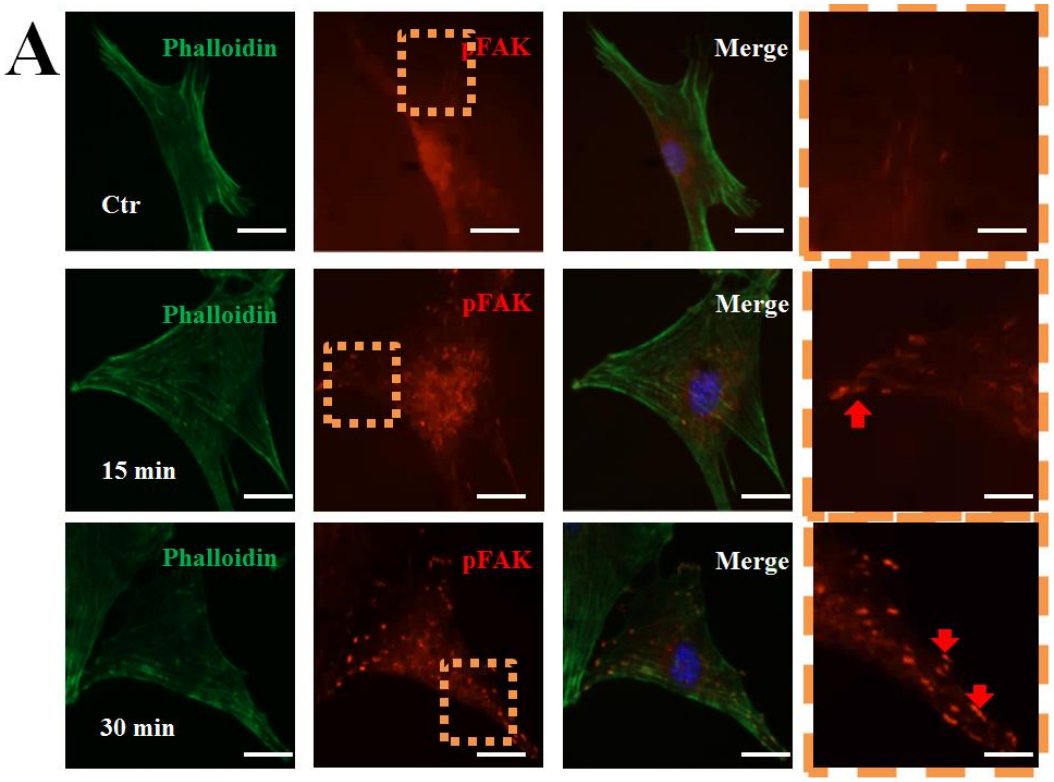


Figure 3.15. Leptin induces the migration of Sca-1$^+$ adventitial progenitor cells by activating the relocation of pFAK. A, Immunofluorescence was performed on Sca-1$^+$ progenitor cells in response to 100 ng/mL of leptin for the detection of pFAK (n=5). Dashed boxes represent magnified fields. Red arrows indicate the activated proteins (scale bars, 3 µm and 1µm). Untreated cells served as a control. Images shown are representative of at least 3 independent experiments.

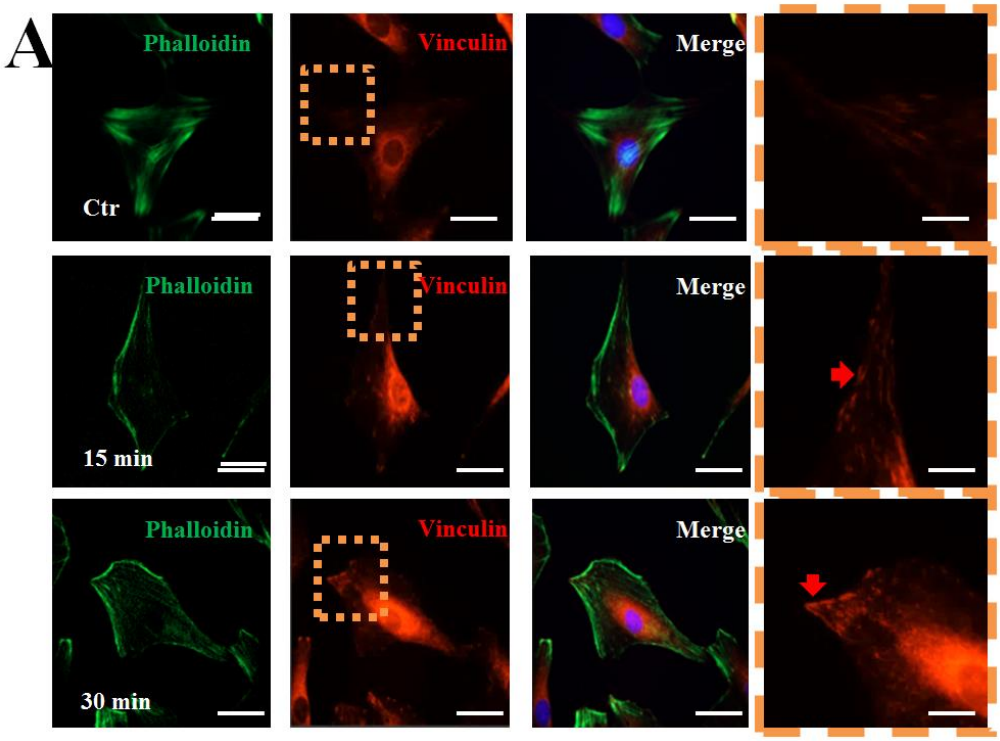


Figure 3.16. Leptin induces the migration of Sca-1^{+} adventitial progenitor cells by activating the relocation of Vinculin. A, Immunofluorescence was performed on Sca-1^{+} progenitor cells in response to 100 ng/mL of leptin for the detection of vinculin (n=5). Dashed boxes represent magnified fields. Red arrows indicate the activated proteins (scale bars, 3 µm and 1µm). Untreated cells served as a control. Images shown are representative of at least 3 independent experiments.

3.7 Involvement of OBR, ERK1/2, and STAT3 in Sca-1$^+$ Progenitor Cells.

So far we proved that OBR, STAT3, ERK1/2, Rho GTPases, pFAK and Vinculin were involved in Sca-1$^+$ progenitor cell response to leptin. However, their interactions remain unknown. Therefore, we pretreated Sca-1$^+$ progenitor cells with different inhibitors, followed by the stimulation of 100ng/mL of leptin. Western blotting revealed that inhibition of the ERK pathway did not affect the activation of pSTAT3 in response to leptin at an early stage (Figure 3.18A, B and C). The inhibition of the STAT3 pathway markedly decreased the expression of pERK1/2 at an early stage, indicating that pERK1/2 is the downstream of pSTAT3 in the OBR-mediated response to leptin (Figure 3.18D, E and F). The late activation of pSTAT3 and pERK at 4 hours (Figure 3.18) after treatment may be caused by the depletion of STAT3 inhibitor or other unknown signaling pathways. OBR deficiency prevented the increase of pSTAT3 with the treatment with 100 ng/mL of leptin, but pERK1/2 was still activated at the late stage (Figure 3.18G, H and I), indicating that other receptors or signaling pathway independent of OBR may be involved in leptin-induced cell response.

So far, OBR was regarded as only functional receptor for leptin. We hence performed a qPCR for the expression of cell migration-related receptors such as CCR 1, 2, 7, 9 and CXCR 3, 4, 5 as it was shown previously that they were upregulated in Sca-1$^+$ progenitor cells during migration (Mei Mei Wong et al., 2013b). Surprisingly, the expression of CXCR5 gene (Figure 3.19) was upregulated 24 hours after the treatment with leptin, indicating its potential role in leptin-induced signaling pathways. We also performed MAPK protein arrays on leptin-stimulated Sca-1$^+$ progenitors. Various phosphorylated MAPK proteins were activated (Figure 3.20) such as p38, CREB, GSK3a, MKK3, RSK1 and 2. However, the potential interactions between CXCR5, MAPK pathway and leptin need further investigation. Taken together, we

confirmed that STAT3 is the upstream of ERK in leptin-induced cell response.

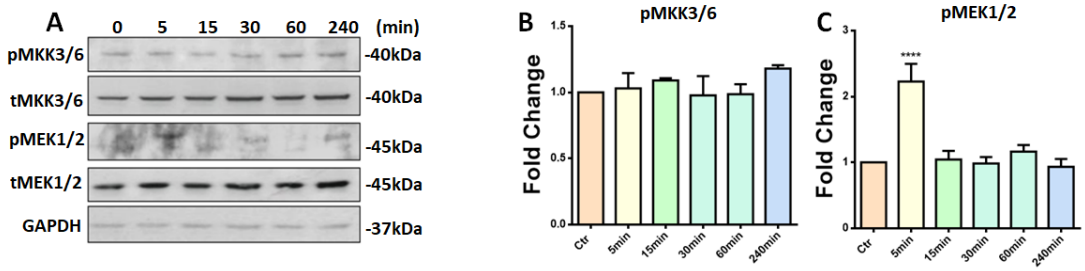


Figure 3.17. Leptin activated pMEK1/2 but not pMKK3/6 signaling pathways on Sca-1⁺ adventitial progenitor cells. A, Western blotting was performed on Sca-1+ progenitor cells in response to 100 ng/ml leptin for the detection of pMKK3/6 (n=3), pMEK1/2 (n=4). **B** and **C**, The quantification of the activation of pMKK3/6 and pMEK1/2. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

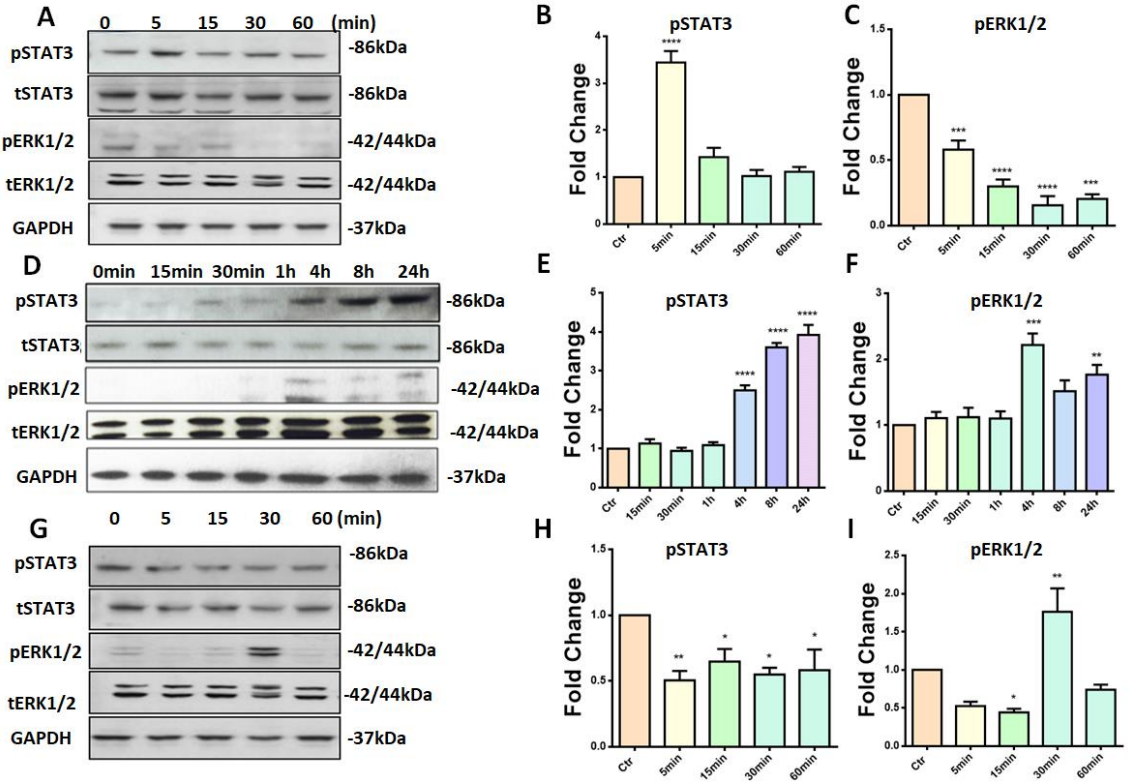


Figure 3.18. OBR and STAT3 were the upstream of ERK1/2. A, Western blotting was performed on Sca-1+ progenitor cells in response to 100 ng/ml leptin with ERK inhibitor for the detection of pSTAT3 (n=8) and pERK1/2

(n=10). **B** and **C**, The quantification of the activation of pSTAT3 and pERK1/2. D, Western blotting was performed on Sca-1+ progenitor cells in response to 100 ng/ml leptin with STAT3 inhibitor for the detection of pSTAT3 (n=4) and pERK1/2 (n=4). **E** and **F**, The quantification of the activation of pSTAT3 and pERK1/2. **G**, Western blotting was performed on Lepr-/-Sca-1+ progenitor cells in response to 100 ng/ml for the detection of pSTAT3 (n=4) and pERK1/2 (n=4). **H** and **I**, The quantification of the activation of pSTAT3 and pERK1/2. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

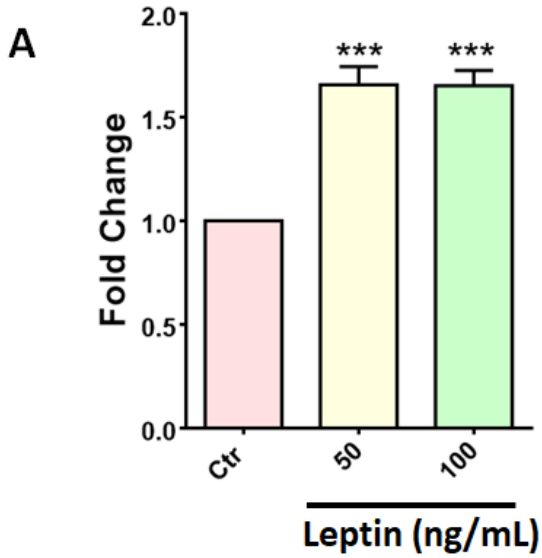
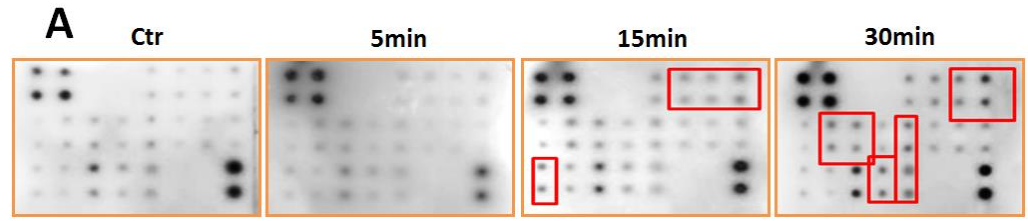


Figure 3.19. Leptin could enhance the expression of CXCR5 . Expression of CXCR5 for Sca-1^{+} progenitor cells was evaluated by qPCR 24 hours after the treatment of leptin (n=5). All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.



Each antibody is spotted in duplicate vertically	A	B	C	D	E	F	G	H
1	POS	POS	NEG	NEG	Akt (P-S473)	CREB (P-S133)	ERK1 (P-T202/Y204) ERK2 (P-Y185/Y187)	GSK3a (P-S21)
2								
3	GSK3b (P-S9)	HSP27 (P-S82)	JNK (P-T183)	MEK (P-S217/221)	MKK3 (P-S189)	MKK6 (P-S207)	MSK2 (P-S360)	mTOR (P-S2448)
4								
5	p38 (P-T180/Y182)	P53 (P-S15)	P70S6K (P-T421/S424)	RSK1 (P-S380)	RSK2 (P-S386)	NEG	NEG	POS
6								

Figure 3.20. Leptin activated various MAPK pathways on Sca-1^{+} adventitial progenitor cells. A, The expression of MAPK-related protein was detected by applying MAPK protein microarrays over time in response to 100 ng/mL of leptin (n=3). Untreated cells were served as control.

3.8 Inhibition of ERK, OBR and STAT3 Could Decrease the Cell Migration

Our results suggested that OBR, STAT3, and ERK1/2 were involved in leptin-induced cell response, but whether these signaling pathways were involved in cell migration or other cell responses remained unclear. Thus, we performed transwell and wound-healing analyses using a $Lepr^{-/-}$ cell model and pathway inhibitors. Inhibitors of leptin receptor, STAT3 and ERK1/2 were applied in the upper chamber of the transwell, while 100 ng/mL of leptin was added into the lower chamber of the transwell. Our results demonstrated that after 16-hour incubation, leptin antagonist CYT-354 (Figure 3.21) and STAT3 inhibitor WP1066 (Figure 3.22) led to a considerable reduction in $Sca1^{+}$ cell migration in transwell assays. Consistent with the results above, inhibition of ERK pathway substantially attenuated the cell migration both in wound-healing (Figure 3.23) and transwell assays (Figure 3.24), especially at a high dose of inhibitors. The migration of $Lepr^{-/-}$ $Sca\text{-}1^{+}$ progenitor cells in both wound-healing (Figure 3.25) and transwell assay (Figure 3.26) was significantly reduced in response to 100 ng/mL of leptin compared to $Lepr^{+/+}$ cells, suggesting that most of leptin-induced cell migration was mediated by leptin receptor b (OBR). In summary, OBR, STAT3 and ERK could participate in the process of migration of $Sca\text{-}1^{+}$ progenitor cells.

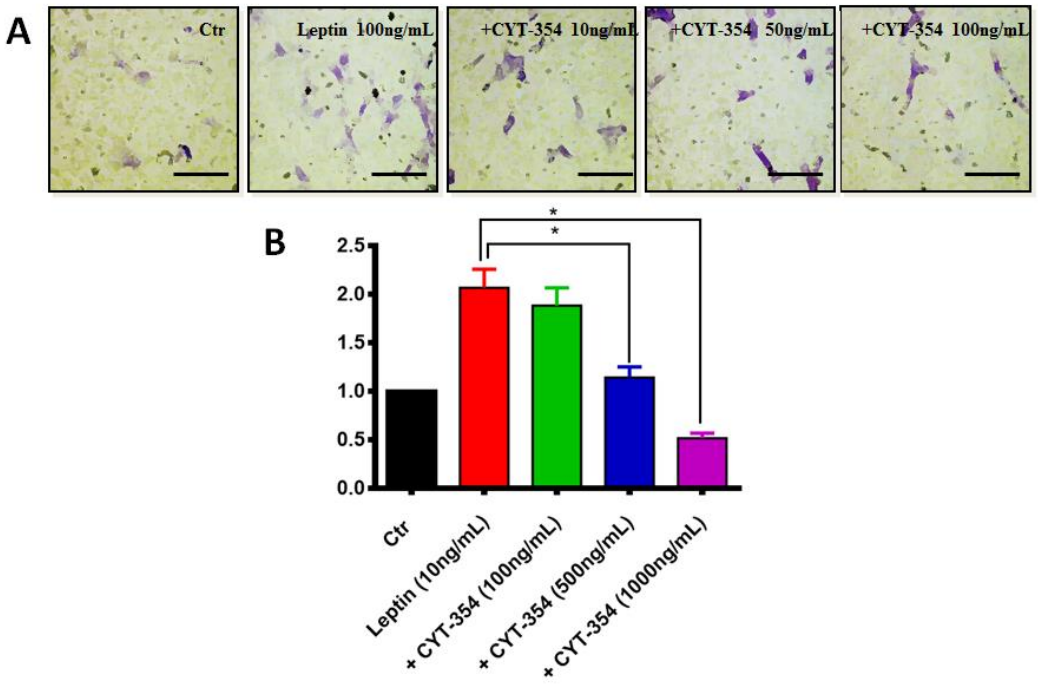


Figure 3.21. Leptin-induced chemotaxis can be abolished by CYT-354 treatment. **A** and **B**, Chemotaxis of Sca-1^{+} progenitor cells in response to 100 ng/mL of leptin and CYT-354 (A) (n=5) in an 8.0 μm transwell system was identified using 1% crystal violet staining after 16-hour incubation (scale bars, 20 μm). Serum-free medium and dimethyl sulfoxide (DMSO) were used as controls. Migration index of transwell assays was defined as the mean ratio of treatment to control of cell numbers counted per 5 random fields at 20X magnification. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

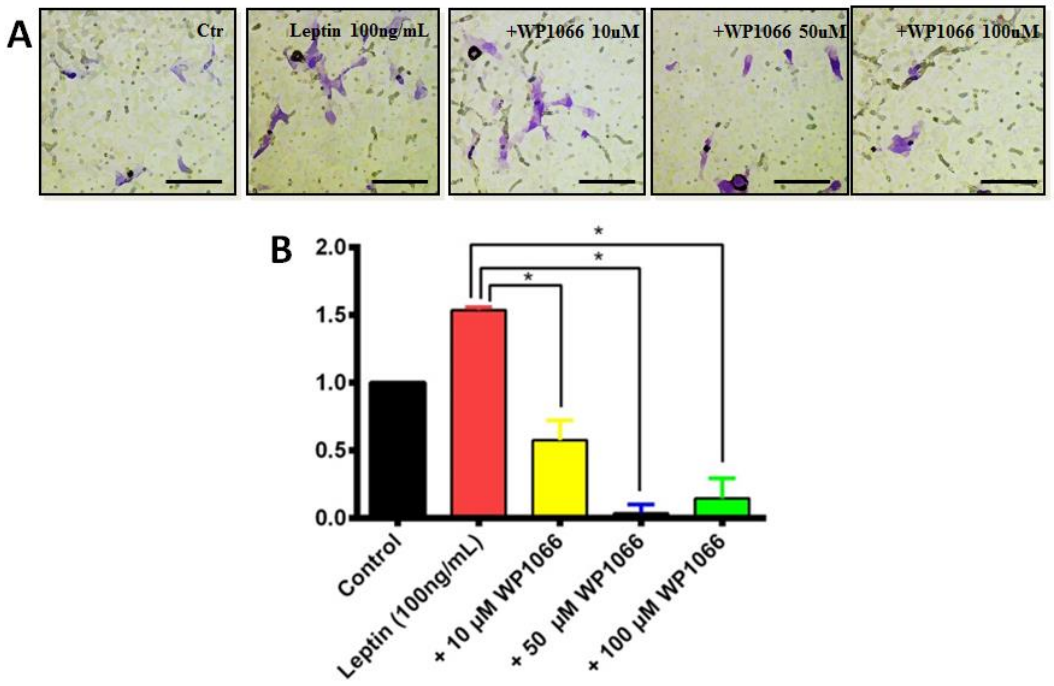


Figure 3.22. Leptin-induced chemotaxis can be abolished by WP1066 treatment. A and **B**, Chemotaxis of Sca-1^{+} progenitor cells in response to 100 ng/mL of leptin and WP1066 (A) (n=5) in an 8.0 μm transwell system was identified using 1% crystal violet staining after 16-hour incubation (scale bars, 20 μm). Serum-free medium and dimethyl sulfoxide (DMSO) were used as controls. Migration index of transwell assays was defined as the mean ratio of treatment to control of cell numbers counted per 5 random fields at 20X magnification. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

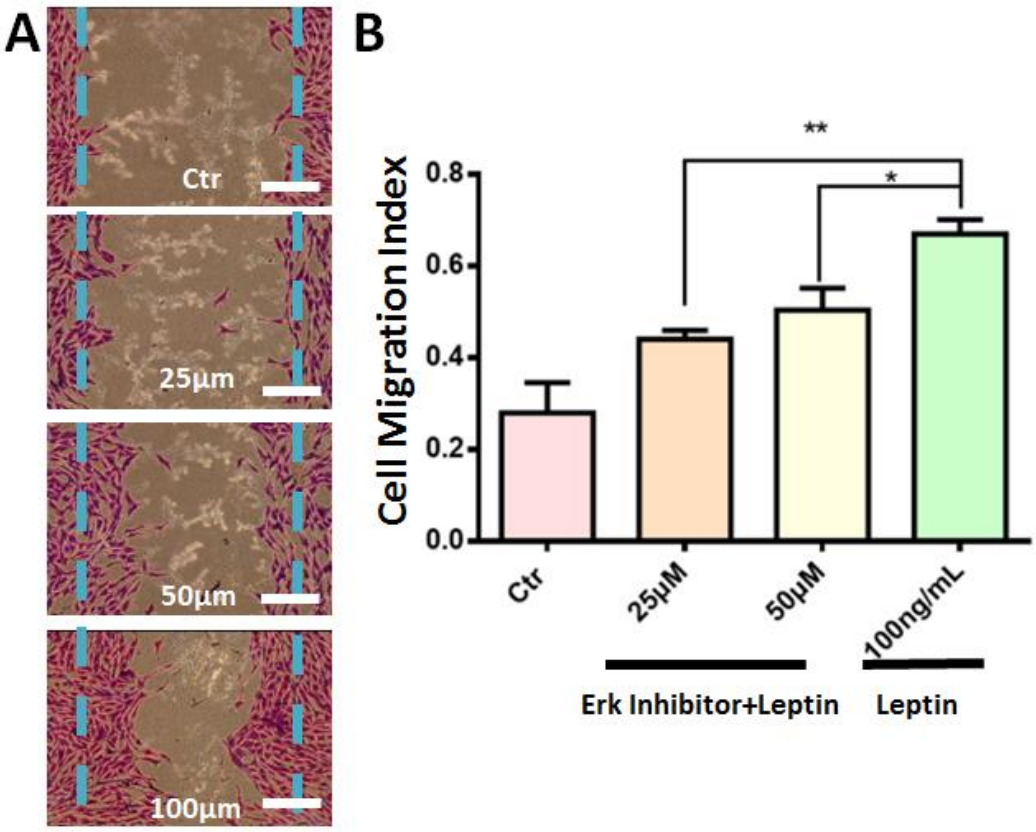


Figure 3.23. Inhibition of ERK pathway significantly reduced the migration of Sca-1$^+$ adventitial progenitor cells in response to 100ng/mL leptin. A and **B**, Migration of Sca-1$^+$ progenitor cells in response to 100 ng/mL of leptin and ERK inhibitor was evaluated by applying 1% crystal violet staining after 16-hour incubation (scale bars, 50 µm, n=5). Migration index for wound healing assays was defined as the mean ratio of treatment to control of migrated area analyzed per 5 random fields at 20X magnification. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

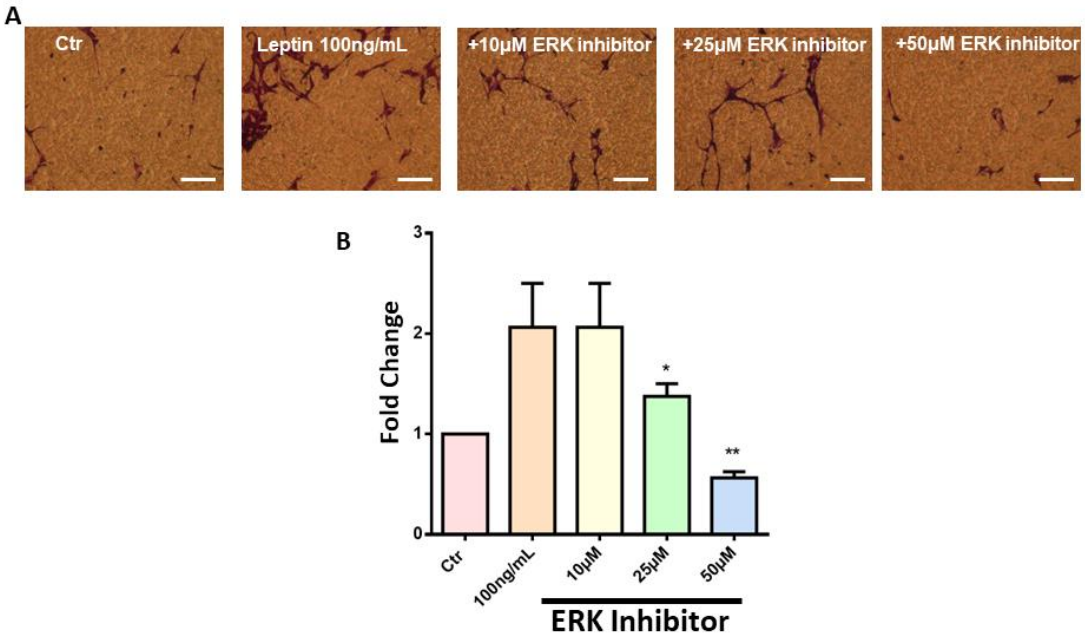


Figure 3.24. Inhibition of ERK pathway significantly reduced the chemotaxis of Sca-1$^+$ adventitial progenitor cells in response to 100ng/mL leptin. A and **B**, Chemotaxis of Sca-1$^+$ progenitor cells in response to 100 ng/mL of leptin and ERK inhibitor was documented by using an 8.0 μm transwell system (scale bars, 50 μm, n=5). Serum-free cultured medium and dimethyl sulfoxide (DMSO) without leptin treatment was used as controls for the migratory assays above. Migration index for transwell assays was defined as the mean ratio of treatment to control of cell numbers counted per 5 random fields at 20X magnification. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

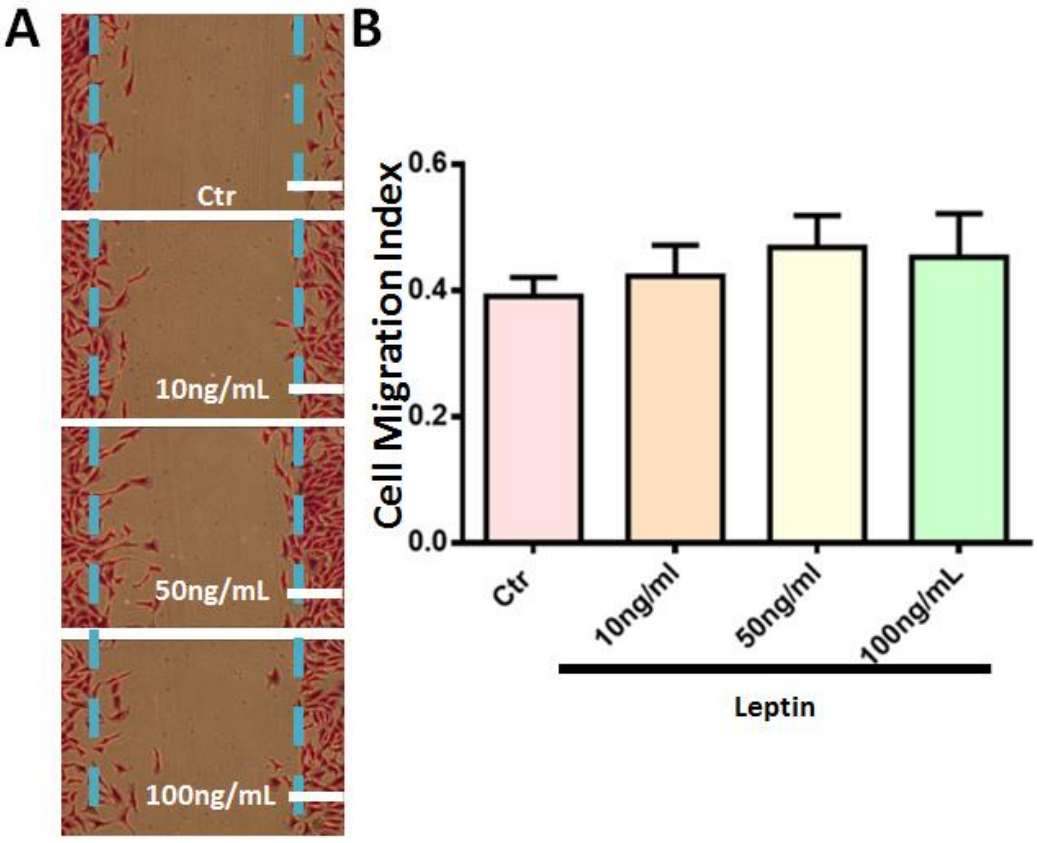


Figure 3.25. Lack of OBR significantly reduced the migration of Sca-1$^+$ adventitial progenitor cells in response to 100ng/mL leptin. A and **B**, Migration of Sca-1$^+$ progenitor cells from db/db mice in response to an increasing gradient concentration of leptin was evaluated by 1% crystal violet staining after 16-hour incubation (scale bars, 50 μm, n=5). Serum-free cultured medium and dimethyl sulfoxide (DMSO) without leptin treatment was used as controls for the migratory assays above. Migration index for wound healing assays was defined as the mean ratio of treatment to control of migrated area analyzed per 5 random fields at 20X magnification. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

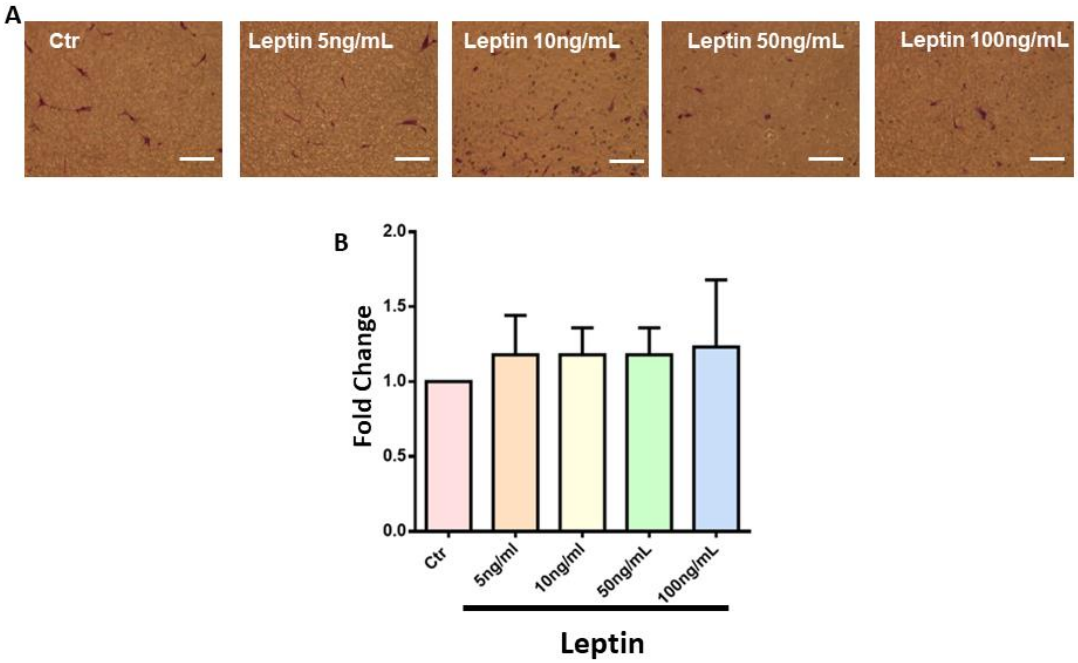


Figure 3.26. Lack of OBR pathway significantly reduced the chemotaxis of Sca-1$^+$ adventitial progenitor cells in response to 100ng/mL leptin. A and **B**, Chemotaxis of Sca-1$^+$ progenitor cells from db/db mice in response to an increasing gradient of leptin in an 8.0 μm transwell system was identified by applying 1% crystal violet staining after 16-hour incubation (scale bars, 50 μm, n=5). Serum-free cultured medium and dimethyl sulfoxide (DMSO) without leptin treatment was used as controls for the migratory assays above. Migration index for transwell assays was defined as the mean ratio of treatment to control of cell numbers counted per 5 random fields at 20X magnification. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

3.9 OBR Is Required for the Migration of Sca-1$^+$ Progenitor Cells *In Vivo*

We investigated the role of OBR in Sca-1$^+$ cell migration *in vivo*, following a guide-wire injury of the femoral artery in mice. Femoral arteries after endovascular injury displayed a very strong autofluorescence at the wavelength of 488 nm, which could make it difficult to track the transplanted GFP cells. Therefore, Sca-1$^+$ progenitor cells were transfected with lentiviral vectors harboring RFP gene to trace live cell migration *in vivo* (Figure 3.27). The RFP Sca-1$^+$ progenitor cells were photographed at the wavelength of 594 nm. FACS assays demonstrated that 91.2% of Sca-1$^+$ progenitor cells successfully expressed RFP (Figure 3.28). Male mice were randomly divided into two groups: one group underwent guide-wire injury of femoral artery, followed by transplantation of RFP Sca-1$^+$ progenitor cells in 30 µL of Matrigel on the adventitial side of the injured vessel. Another group underwent the same procedure, but additionally CYT-354 (Leptin antagonist) was mixed with the Matrigel. One or three days after surgery, the injured femoral arteries were harvested, and *En Face* staining was performed. Our data demonstrated that Sca-1$^+$ progenitor cells could migrate from the adventitia to intima at a very early stage (Figure 3.29Aa, Ab, 3.30A, B and C). Three days after the endovascular injury, Sca-1$^+$ progenitor cells were discovered in the media layer of femoral arteries (Figure 3.31A). When the leptin inhibitor CYT-354 was added in Matrigel®, Sca-1$^+$ progenitor cells migration was significantly alleviated, indicating the substantial effect of leptin and OBR on cell migration *in vivo* (Figure 3.29Ac, Ad, 3.30A, B, and D). We also transplanted Sca-1$^+$ progenitor cells on the femoral arteries of db/db mice. It was well known that db/db mice were atherosclerosis protective (Stephenson et al., 2003). Surprisingly, the migratory cells were also observed in injured arteries three days post-surgery (Figure 3.31B, and 3.28 C) although the number of migratory cells was reduced in comparison to what was observed

in wild-type mice (Figure 3.32A).

Additionally, a population of migratory RFP Sca-1$^+$ progenitor cells acquired SMC markers (Figure 3.33A) and lost Sca-1 marker expression (Figure 3.33B) during the migration. It revealed the possibility that these cells may undergo differentiation *in vivo* before they reach the intima. Therefore, we also assessed the change of SM22α and calponin expression in Sca-1$^+$ progenitor cells in response to 100 ng/mL of leptin. Our data showed that leptin could potentially induce cell differentiation after 24-hour treatment (Figure 3.34). However, full mechanisms of cell differentiation needs further investigation.

Next, we applied Lepr$^{-/-}$ Sca-1$^+$ progenitor cells on the injured arteries of wild-type mice and found that very few RFP$^+$ cells had migrated inside the artery (Figure 3.31C, 3.32A), the reason of which may be related to lack of leptin-induced cell migration on Lerp$^{-/-}$ Sca-1$^+$ progenitors. These results demonstrated that the OBR on Sca-1$^+$ progenitor cells plays a key role in cell migration from adventitia to intima after endothelial injury. Indeed, lack of OBR substantially abolished cell migration.

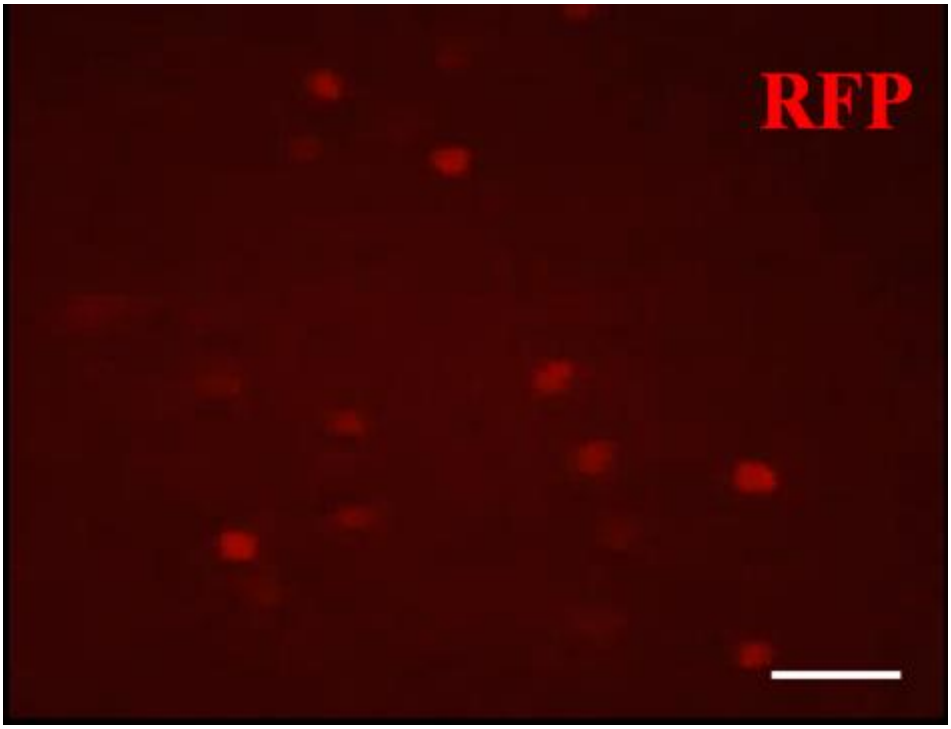


Figure 3.27. RFP-Sca-1-APC cells showed red fluorescence *in vitro*. Sca-1^{+} progenitor cells were successfully transfected by RFP lentivirus (scale bars, 40 μm).

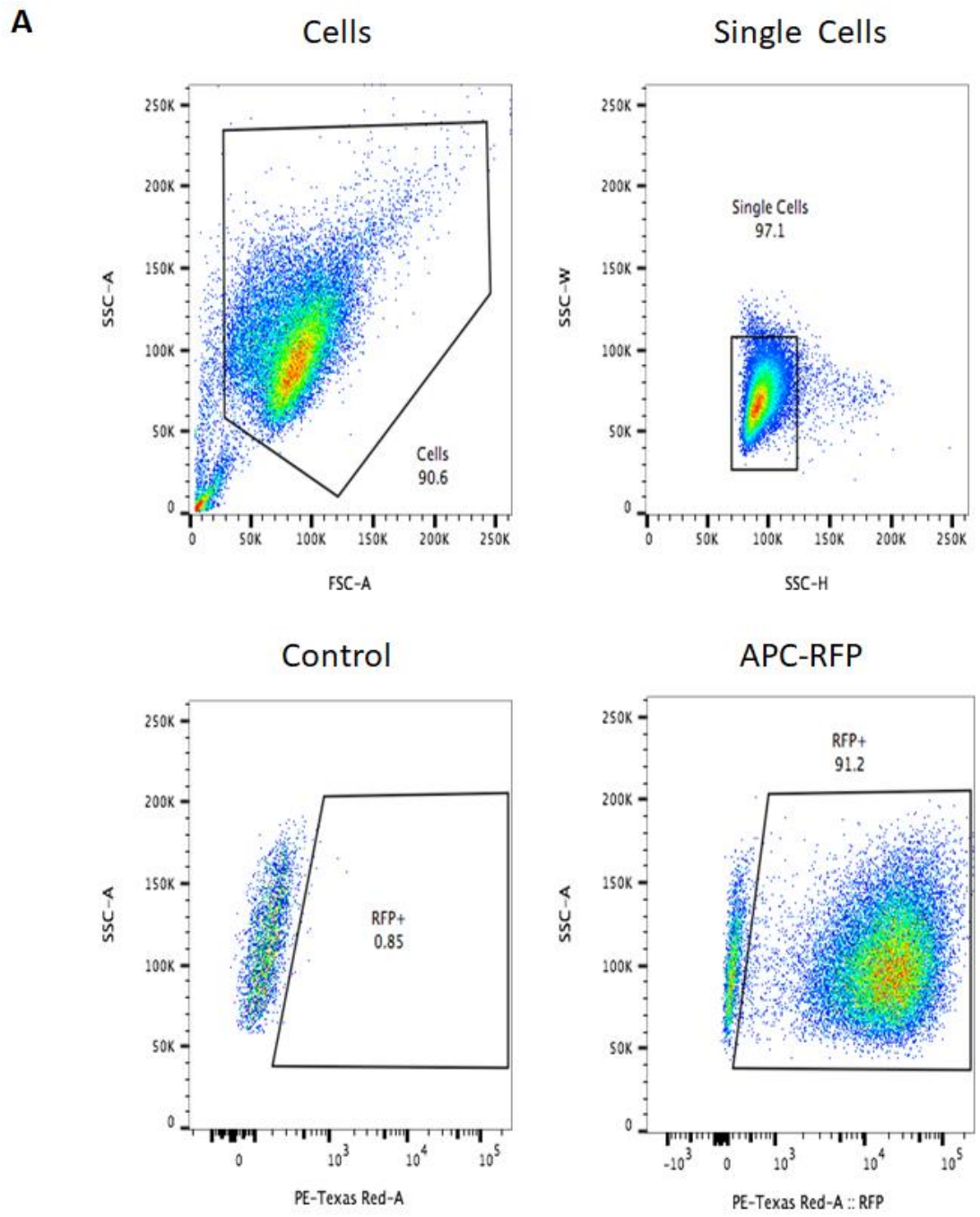


Figure 3.28. Identification of RFP and non-RFP Sca-1$^+$ adventitia progenitor cells after lentivirus transfection. A, Confirmation of the existence of RFP in Sca-1$^+$ adventitia progenitor cells with primary antibodies of RFP by applying FACS assay.

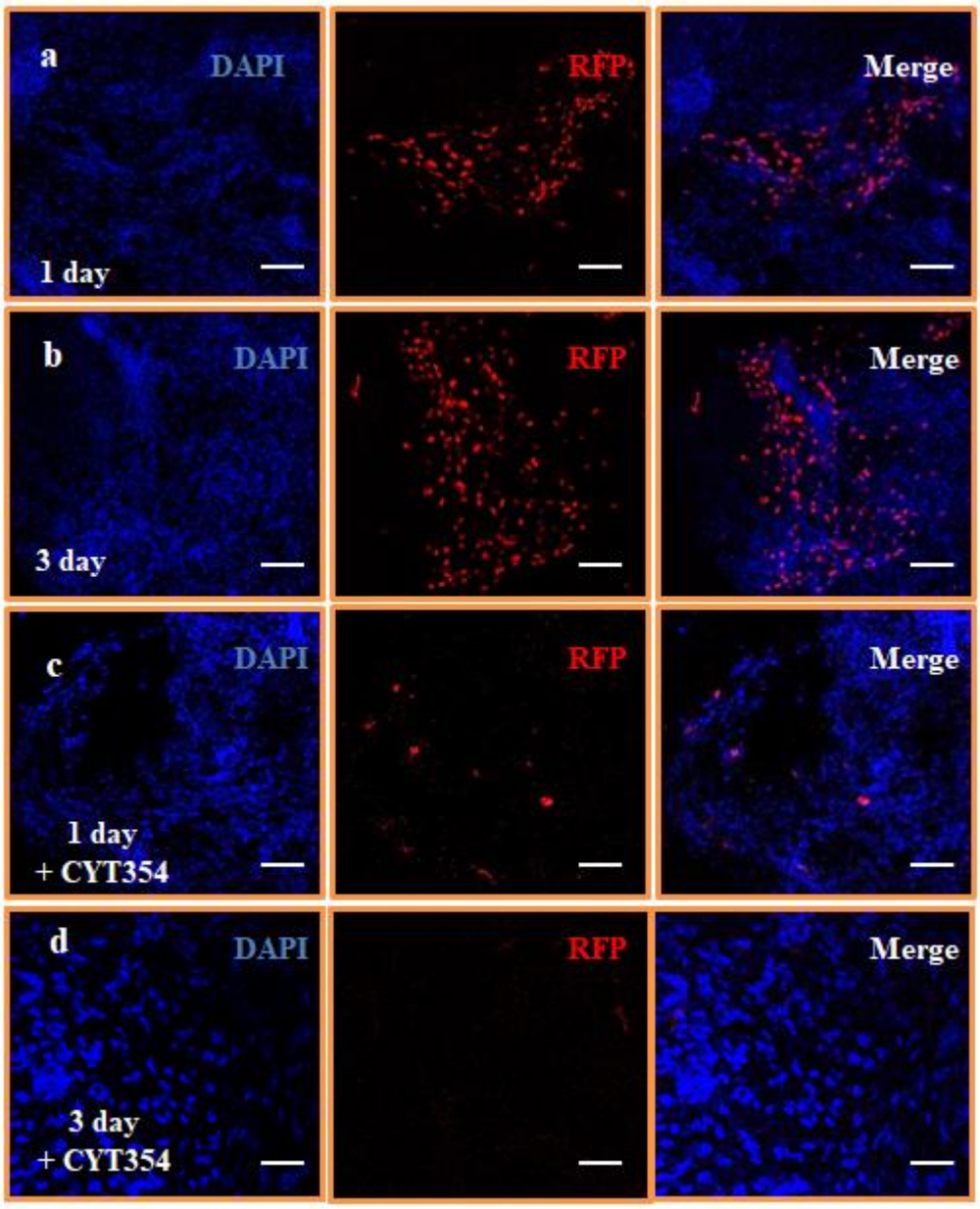


Figure 3.29. Sca-1^{+} progenitor cells could migrate towards intima on 1 and 3 days post-surgery. A, 1*10^{6} RFP Sca-1^{+} with (c and d, scale bar, 50 μm) or without CTY-354 (a and b, scale bar, 100 μm) were seeded in the adventitial side of injured femoral arteries (n=6). *En face* staining showed that the RFP cells migrated from the adventitia to the intima on day 1 or 3 post-surgery. Cell migration assay *in vivo* was performed in wild-type mice.

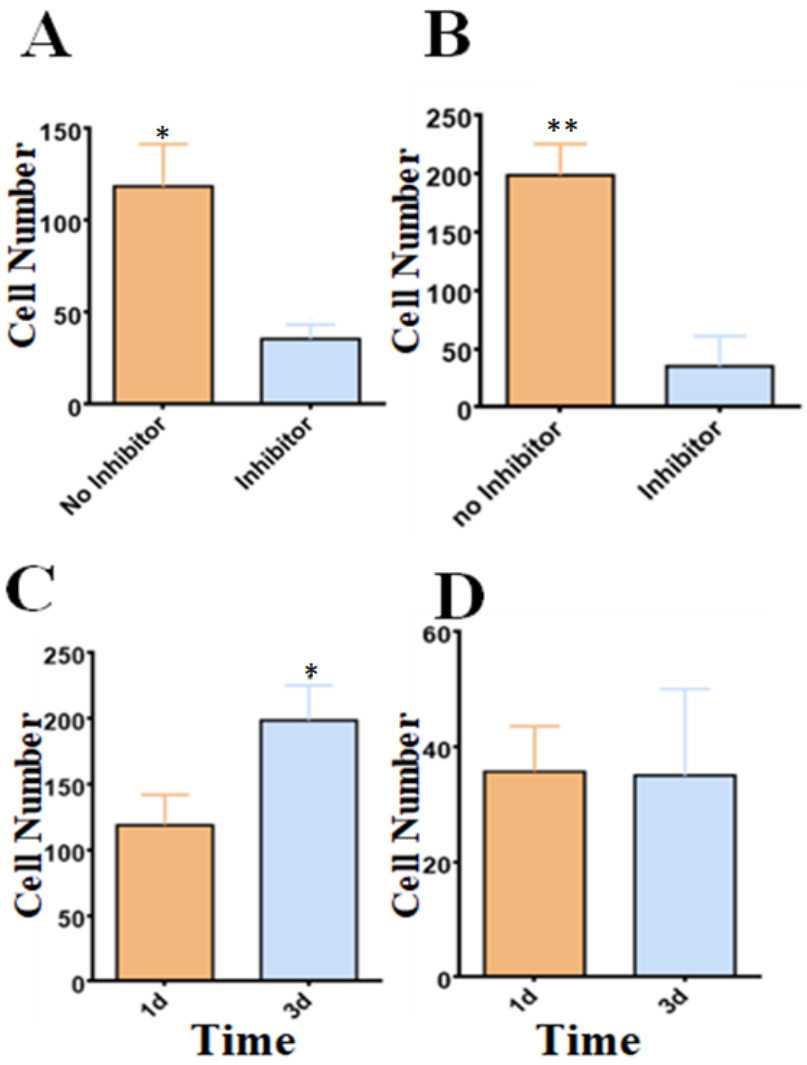


Figure 3.30. Leptin-induced migration of Sca-1$^+$ progenitor cells can be abolished by CYT-354 treatment *in vivo*. **A**, Quantification of migratory cells from the adventitia to intima with or without CYT-354 inhibitor on day 1 post-surgery. **B**, Quantification of migratory cells from the adventitia to intima with or without CYT-354 inhibitor on day 3 post-surgery. **C**, Quantification of migratory cells from the adventitia to intima without CYT-354 inhibitor on day 1 or 3 post-surgery. **D**, Quantification of migratory cells from the adventitia to intima with CYT-354 inhibitor on day 1 or 3 post-surgery.Cell migration assay *in vivo* was performed in wild-type mice. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

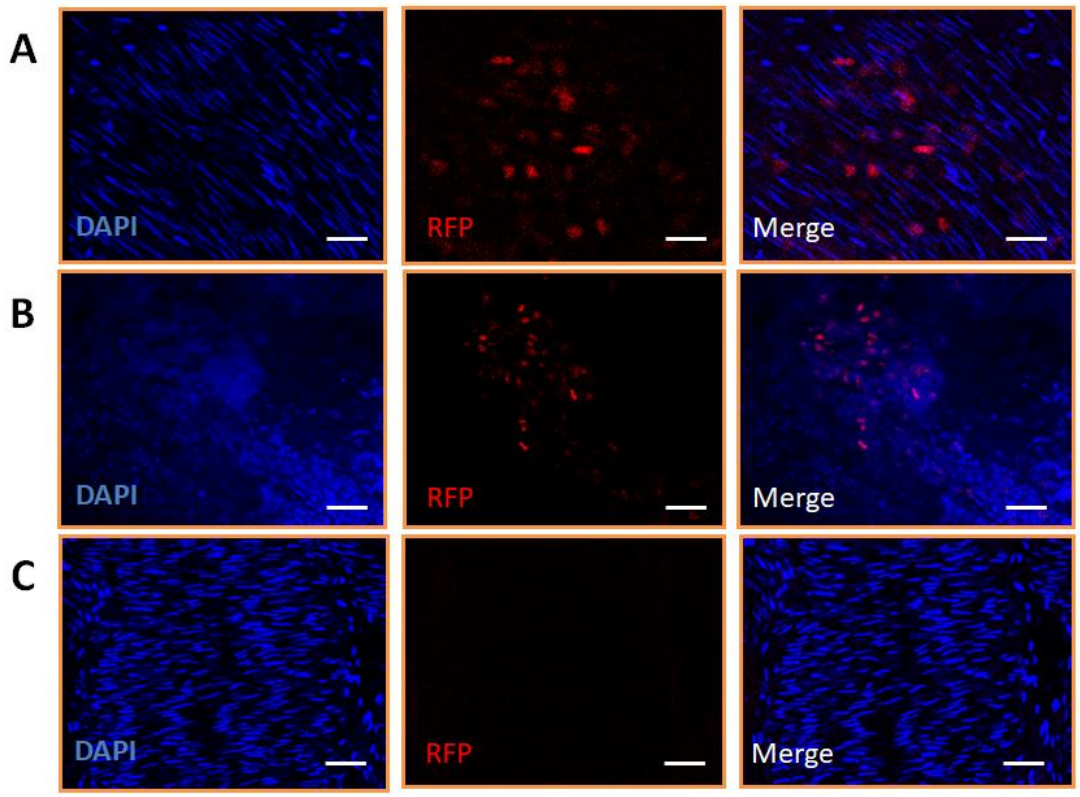


Figure 3.31. Leptin receptor played an important role in cell migration *in vivo.* **A**, Cell migration assay *in vivo* was performed in wild-type mice. $1*10^6$ RFP Sca-1^+ were seeded in the adventitial side of injured femoral artery in wild-type mice. *En face* staining showed that the RFP cells migrated towards the intima at 72 hours after the surgery (scale bar, 30 μm, n=6). **B**, $1*10^6$ RFP Sca-1^+ were seeded in the adventitial side of injured femoral artery in db/db mice. *En face* staining showed that the RFP cells migrated towards the intima at 72 hours after the surgery (scale bar, 100 μm, n=6). **C**, $1*10^6$ RFP $lepr^{-/-}$ Sca-1^+ were seeded in the adventitial side of injured femoral artery in wild-type mice. *En face* staining showed that the RFP cells did not migrate towards the intima at 72 hours after the surgery (scale bar, 50 μm, n=6).

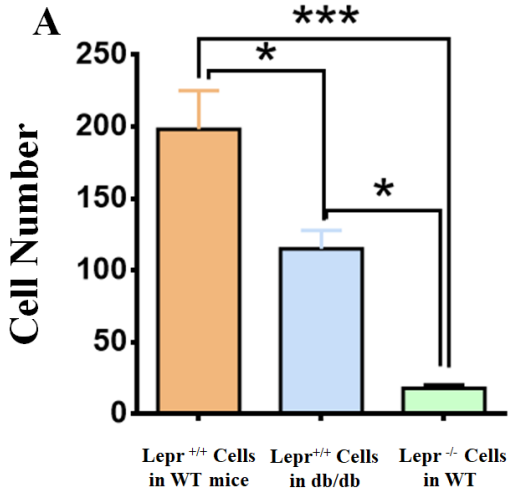


Figure 3.32. Lack of leptin receptor in Sca-1⁺ progenitor cells abolished the leptin-induced cell migration *in vivo*. A, Quantification of migratory cells from the adventitia for group A, B and C in Figure 3.27. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

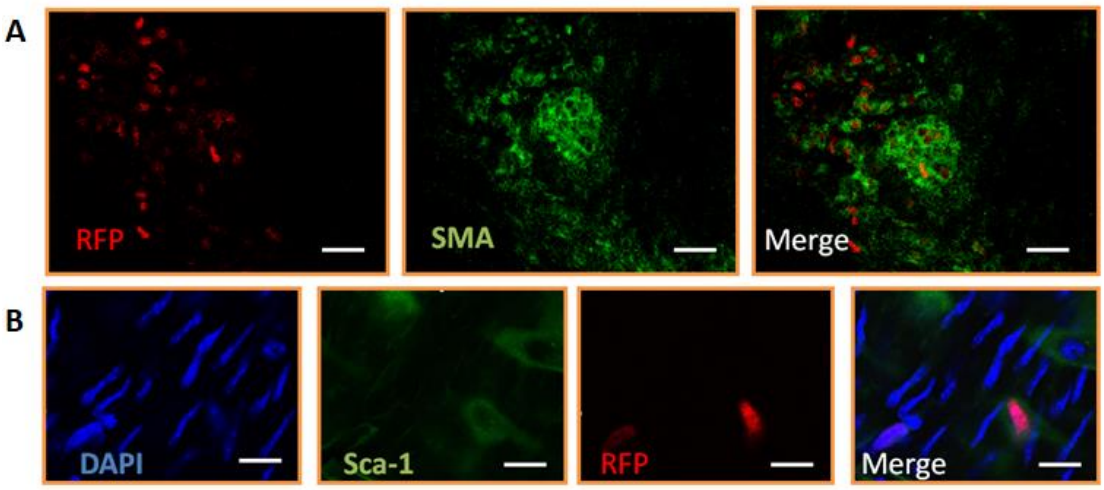


Figure 3.33. Sca-1⁺ progenitor cells underwent cell differentiation *in vivo*. A, $1*10^6$ RFP Sca-1⁺ cells were seeded in the adventitial side of injured femoral artery in db/db mice. *En face* staining showed that the RFP cells acquired smooth muscle cell marker (Alexa 488; green) at 72 hours after the surgery (scale bar, 20 μm, n=6). **B**, $1*10^6$ RFP Sca-1⁺ cells were seeded in the adventitial side of injured femoral artery in wild-type mice. *En face* staining showed that some RFP cells lost Sca-1⁺ marker (Alexa 488; green) at 72 hours after the surgery (scale bar, 30 μm, n=6).

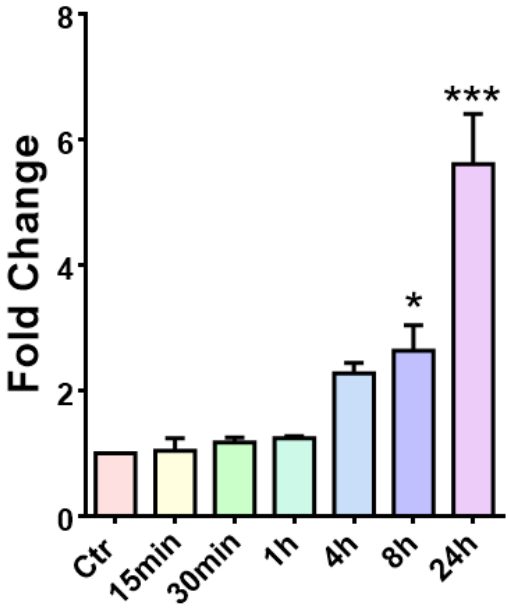


Figure 3.34. Leptin may induce the differentiation of Sca-1^{+} progenitor cells towards SMC. A, Expression of Calponin (n=5) for Sca-1+ progenitor cells was evaluated by qPCR in response to 100 ng/mL leptin. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

3.10 Migration of Sca-1$^+$ Progenitor Cells Is Related to Elevated Serum Leptin

Data presented so far indicated that Sca-1$^+$ progenitor cells could migrate into the injured artery and the absence of OBR could diminish this effect. However, the factor responsible for Sca-1$^+$ progenitor migration into the intima was still unknown. As leptin could induce Sca-1$^+$ progenitor cell migration *in vitro*, we wondered whether the concentration of leptin was also enhanced after endovascular injury. Therefore, we performed an ELISA to measure serum leptin level on day 1, 7 and 14 after the guide-wire injury and assessed whether leptin could play a similar role *in vivo*. Our data revealed that serum leptin increased one day post-surgery (Figure 3.35). This upregulation could be the inducer of Sca-1$^+$ progenitor cells migration *in vivo* post-surgery. In addition, db/db mice showed a much higher concentration of serum leptin (Figure 3.36) which may explain the Sca-1$^+$ cell migration we observed in db/db mice (Figure 3.31B).

Also, we detected a higher immunostaining for leptin in cells of the vessel wall (Figure 3.38B) one day post-surgery compared to the immunostaining in an uninjured artery (Figure 3.38A). Western blotting for the whole artery revealed that expression of leptin was upregulated one day after the guide-wire injury (Figure 3.37). Since the expression of leptin seemed to originate from smooth muscle cells in medial layer (Figure 3.38B), we also performed immunostaining for SMC with leptin primary antibody. We confirmed that SMCs could express leptin *in vitro* under normal culturing conditions (Figure 3.39), providing another possible origin of leptin *in vivo*. These data, taken together, demonstrated that the expression of leptin was enhanced both systemically and locally after the endovascular injury.

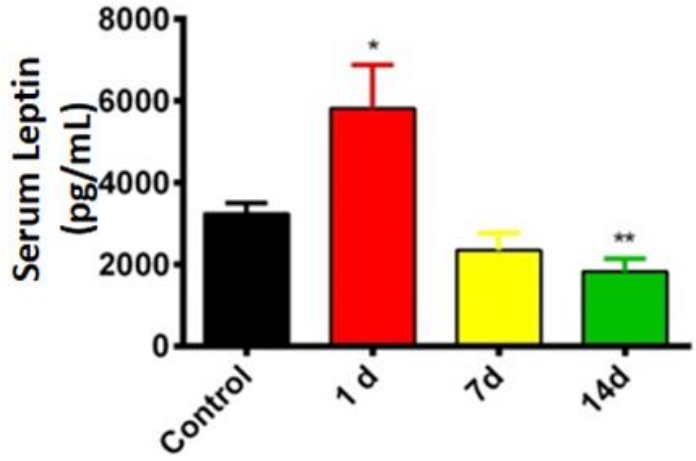


Figure 3.35. Serum leptin was upregulated on 1 day after surgery. Serum leptin in wild-type mice on day 1 (n=10), 7 (n=5) and 14 (n=12) after injury were quantified by using leptin ELISA kit. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

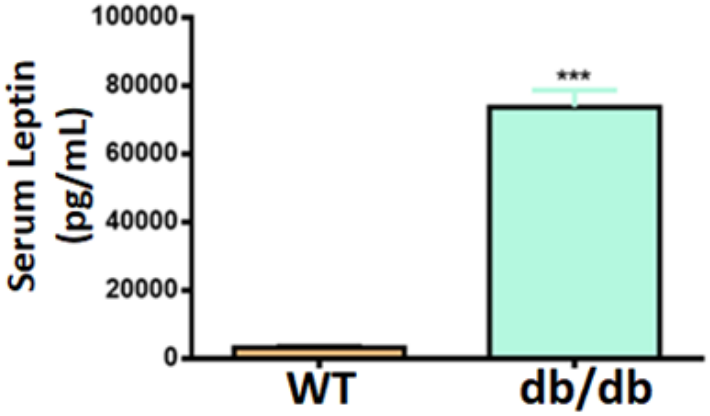


Figure 3.36. Serum leptin was much higher in db/db mice. Serum leptin in wild-type and db/db mice was quantified by using leptin ELISA kit. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

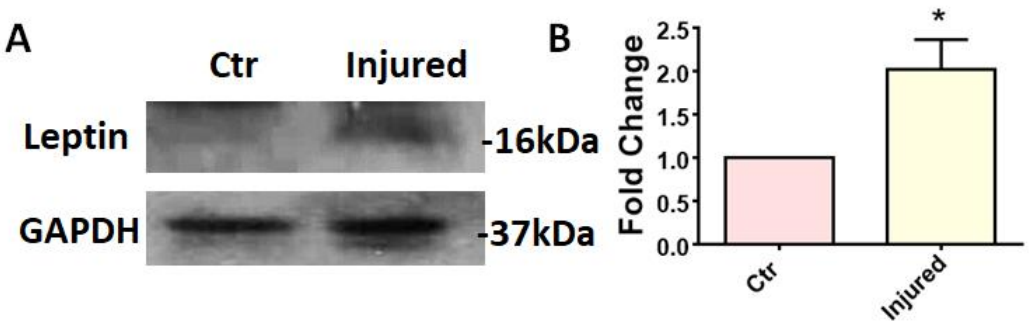


Figure 3.37. The expression of leptin was upregulated after surgery. A, Expression of leptin in the injured or non-injured arteries was documented by performing western blotting one day post-surgery (n=4). **B**, Quantification of leptin expression in non-injured and injured vessels. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

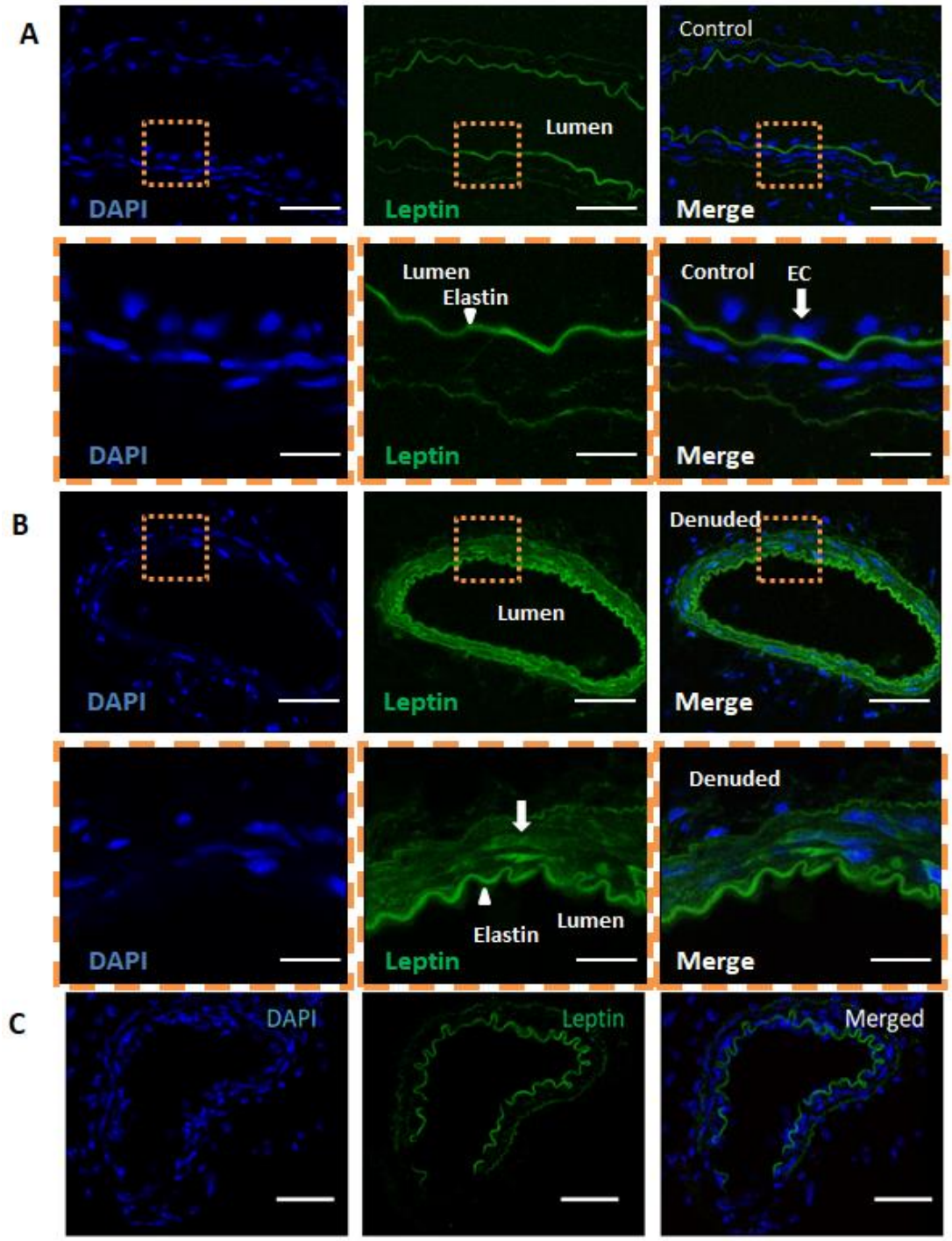


Figure 3.38. The expression of leptin was upregulated in the injured artery. A and **B**, Difference in expression of leptin (Alexa 488; green) in non-injured (A) artery (n=6) and injured (B) artery (n=10) was analyzed on 1 day post-suegery by immunofluorescence (scale bars, 30 and 7 μm). **C**, Negative control for leptin staining (scale bars, 30 μm).

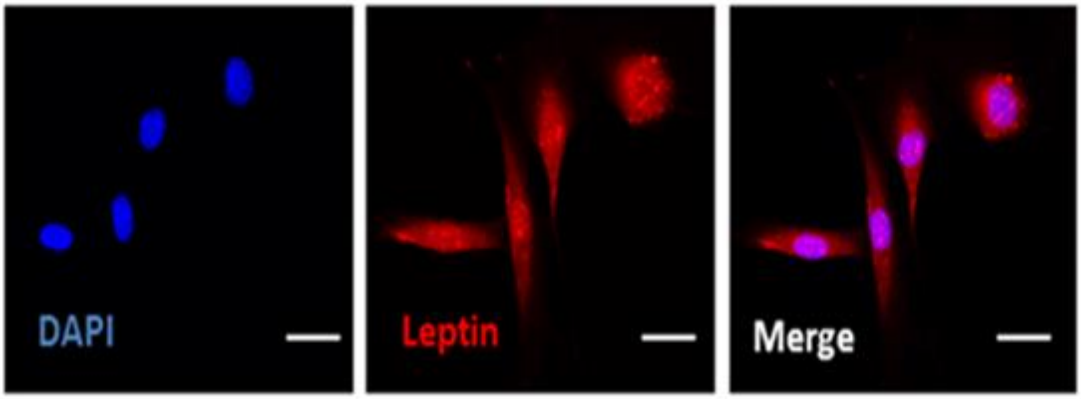


Figure 3.39. Smooth muscle cells showed expression of leptin *in vitro*. Expression of leptin in smooth muscle cell (Alexa 594; red) *in vitro* was detected by immunofluorescence (scale bars, 8 μm).

3.11 Migration of Sca-1$^+$ Progenitor Cells Contribute to Neointimal Formation

We so far confirmed that OBR played an important role in the migration of Sca-1$^+$ progenitor cells both *in vivo* and *in vitro*, especially in a short-term period. To explore the long-term role of Sca-1$^+$ progenitor cells in neointimal formation, guide-wire injury was performed in wild-type mice. Male mice were randomly separated into two groups: one group underwent guide-wire injury only; while another group underwent an additional Sca-1$^+$ cell transplantation in 30 μL of Matrigel immediately after the injury. The injured arteries were collected either 2 or 4 weeks post-surgery. Histological and morphometric analyses, using paraffin sections of injured femoral arteries, were performed. Wild-type mice in the injury model developed moderate neointimal formation two weeks after the injury, resulting in minor artery narrowing (Figure 3.40 and 3.41A). Neointimal lesions grew significantly in four weeks post-surgery along with a significant reduction in media area (Figure 3.40 and 3.41B to E). The intimal layer increased markedly in both area and thickness, resulting in a high intima-media ratio four weeks after injury (Figure 3.41D). Immunohistochemistry (Figure 3.40B) and immunofluorescence (Figure 3.40C) for αSMA showed that the main component of neointimal formations was smooth muscle cells. Hyperleptinemia has been related to the recruitment of leukocytes and macrophages to the endothelial wall at the early stage of atherosclerosis(Yamagishi et al., 2001). Immunostaining for F4/80 revealed that macrophages started to infiltrate the injured artery from 5 days post-surgery (Figure 3.42C). Moreover, we observed that F4/80 positive cells in the neointima 7 days post-surgery in wild-type mice (Figure 3.42D). The data from wild-type mice with guide-wire injury only were used as a surgery control for further comparison to other groups with different treatments.

Transplantation of Sca-1$^+$ progenitor cells significantly augmented

neointimal formation in wild-type mice 2 weeks post-surgery (Figure 3.44Ad, 3.45 A to D) in comparison to the mice with injury only (Figure 3.45D). This increase in neointimal formation could be abolished when the cells were transplanted with 1000 ng/mL of leptin antagonist CYT-354 (Figure 3.44Aa, Ac, 3.45 A to C). Van Gieson staining revealed that there was no significant difference of the development of fibrosis after the transplantation of exogenous Sca-1$^+$ progenitor cells (figure 3.43). In short, transplanted Sca-1$^+$ progenitor cells could significantly enhance neointimal formation two weeks after surgery, but the administration of CYT-354 inhibited the formation of neointima (Figure 3.45D), indicating that leptin and OBR in Sca-1$^+$ progenitor cells are crucial during the development of neointimal formation after guide-wire injury.

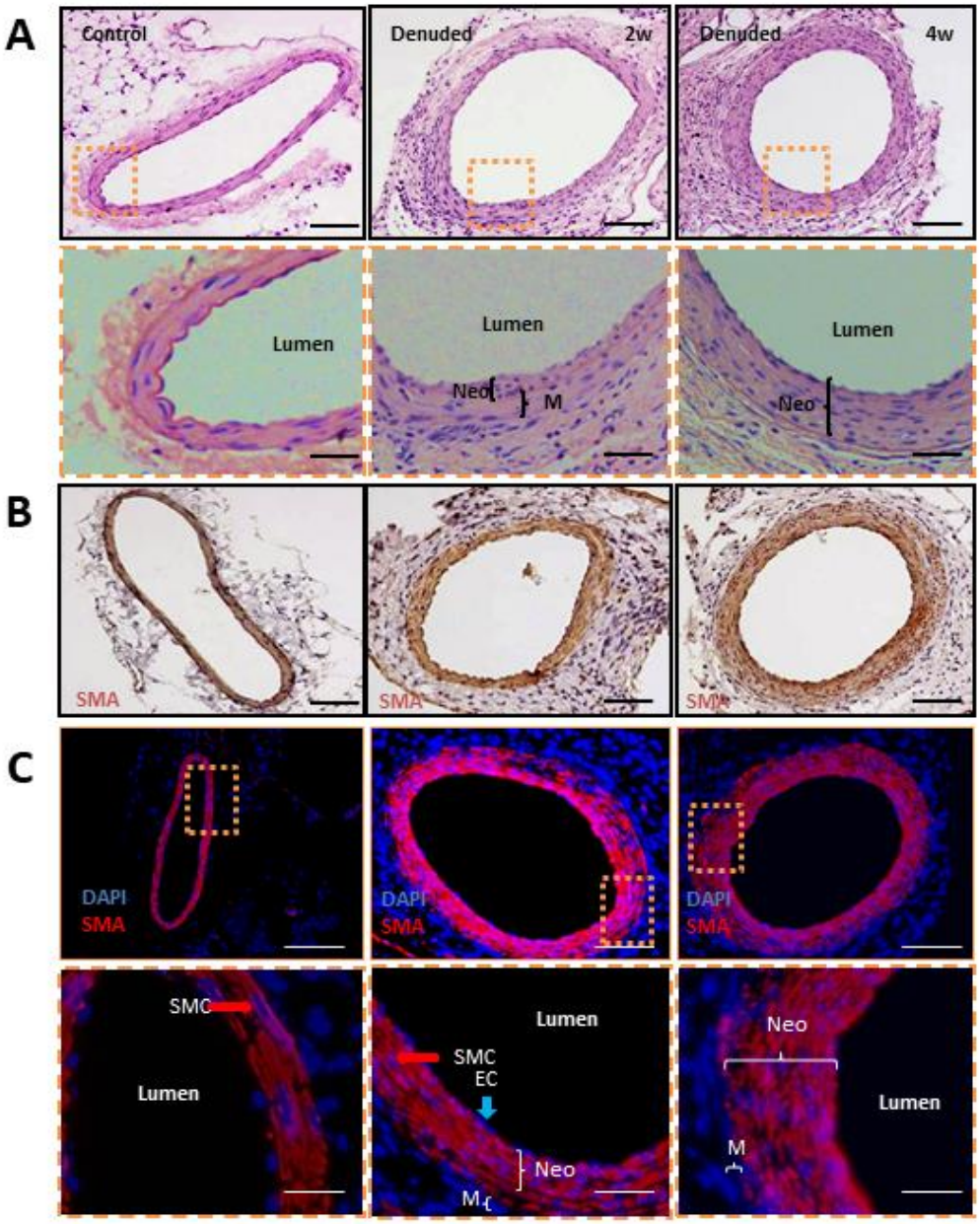


Figure 3.40. Guide-wire injury developed significant neointimal formation. A, H&E staining of the femoral artery after guide-wire injury was performed (scale bar, 50 and 10 μm, n=10). **B** and **C**, The composition of the neointima was evaluated by immunohistochemistry (B) and immunofluorescence (C) for smooth muscle actin on frozen sections of femoral artery at 2 or 4 weeks after surgery (scale bar, 50 and 10 μm, n=10). Dashed box represented the magnified field. Arrows indicate SMCs or ECs. EC indicates endothelium; SMC, smooth muscle cells; Neo, neointima; M, media; SMA, smooth muscle actin; 2W, 2 weeks; 4W, 4 weeks.

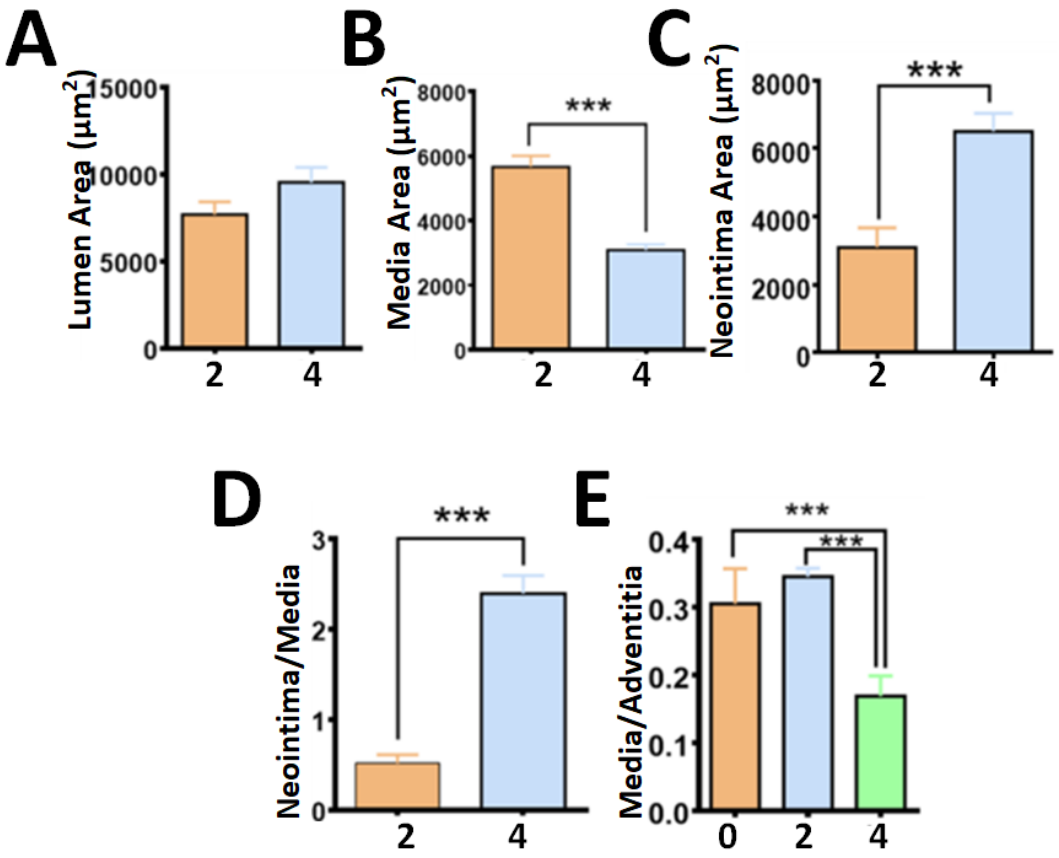


Figure 3.41. Guide-wire injury induced significant neointimal formation 4 weeks after surgery. A, Quantification of lumen area of injured arteries (n=10). **B**, Quantification of media area of injured arteries (n=10). **C**, Quantification of neointimal area of injured arteries (n=10). **D**, Quantification of ratio of neointima to media of injured arteries (n=10). **E**, Quantification of ratio of media to adventitia of controls and injured arteries (n=10). Graphs are shown as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001. EC indicates endothelium; SMC, smooth muscle cells; Neo, neointima; M, media; SMA, smooth muscle actin; 2W, 2 weeks; 4W, 4 weeks.

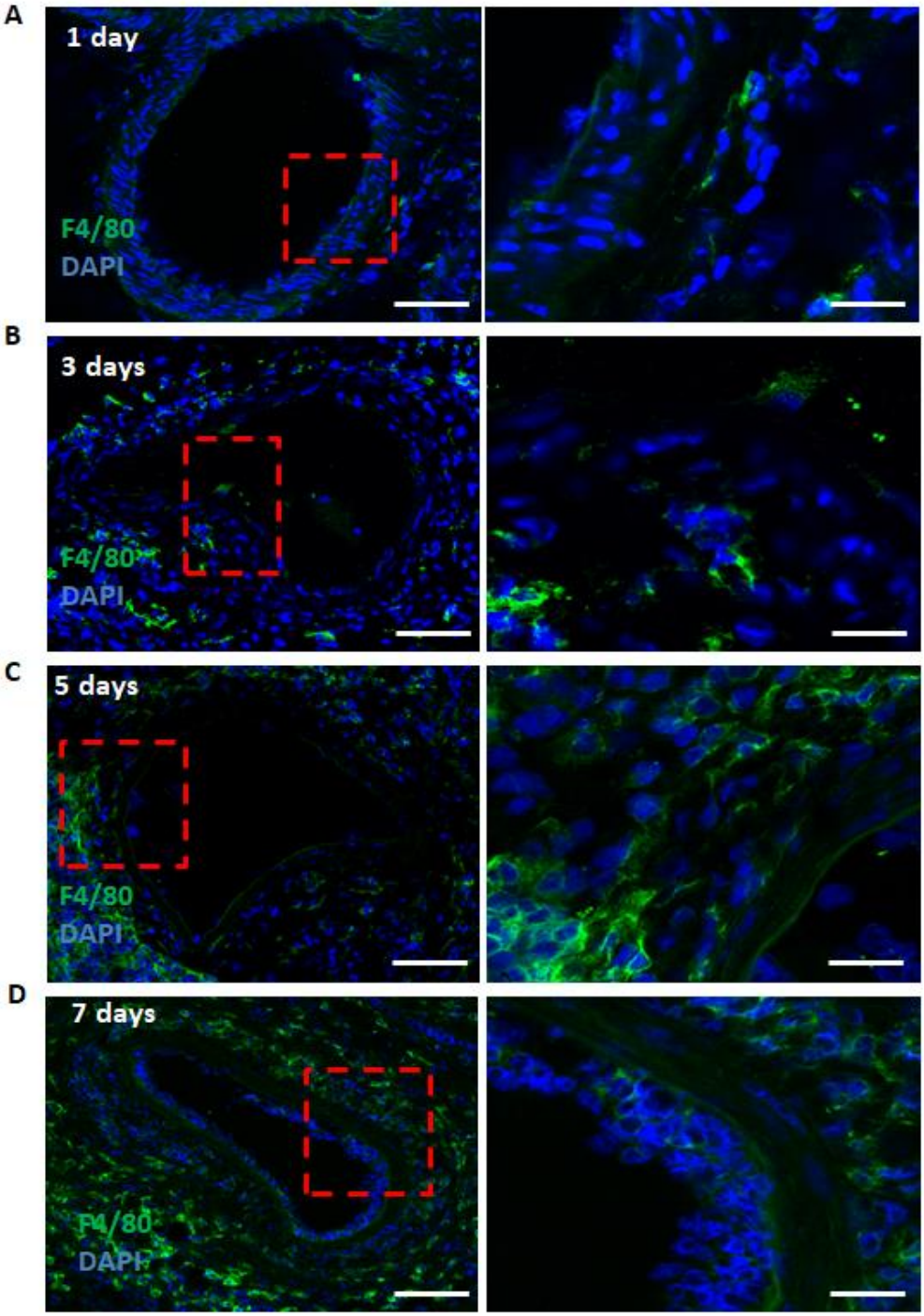


Figure 3.42. The expression of macrophage marker F4/80 was upregulated after surgery around the injured artery. Expression of macrophage (Alexa 488; green) in injured artery was analyzed on 1(A, n=10), 3(B, n=7), 5(C, n=5) and 7(D, n=10) days post-operation by performing immunofluorescence. (scale bars, 50 and 10 μm). Dashed box represented the magnified field.

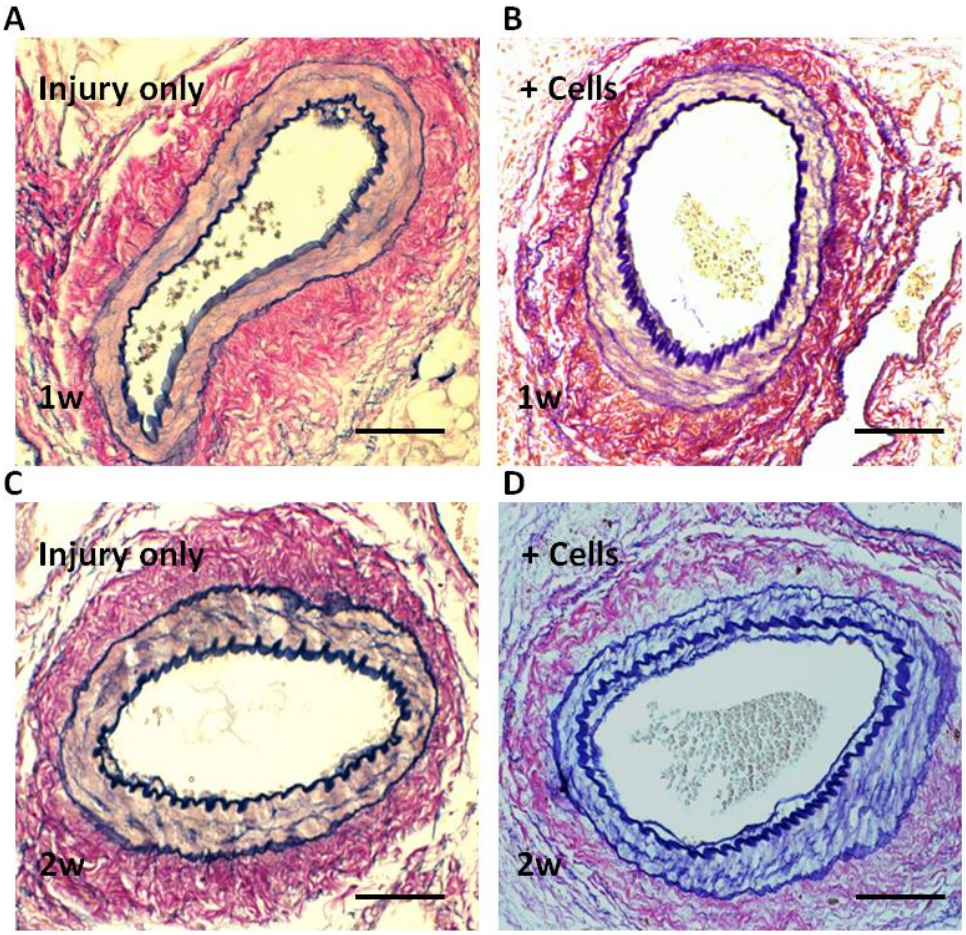


Figure 3.43. Transplantation of exogenous Sca-1^{+} progenitor cells did not affect the extent of firbosis during the neointimal formation. A, Cross sections of injured femoral artery at 1 week post-surgery were analyzed by Elastica-van Gieson staining (scale bars, 50 and 10 μm, n=3). **B**, Lepr$^{+/+}$ Sca-1^{+} progenitor cells were seeded on the adventitial side of injured artery in wild-tpye mice. Cross sections of femoral artery at 1 week post-surgery were analyzed by Elastica-van Gieson staining (scale bars, 50 and 10 μm, n=3). **C**, Cross sections of injured femoral artery at 2 week post-surgery were analyzed by Elastica-van Gieson staining (scale bars, 50 and 10 μm, n=3). **D**, Lepr$^{+/+}$ Sca-1^{+} progenitor cells were seeded on the adventitial side of injured artery in wild-tpye mice. Cross sections of femoral artery at 2 week post-surgery were analyzed by Elastica-van Gieson staining (scale bars, 50 and 10 μm, n=3). Images shown are representative of at least 3 independent experiments.

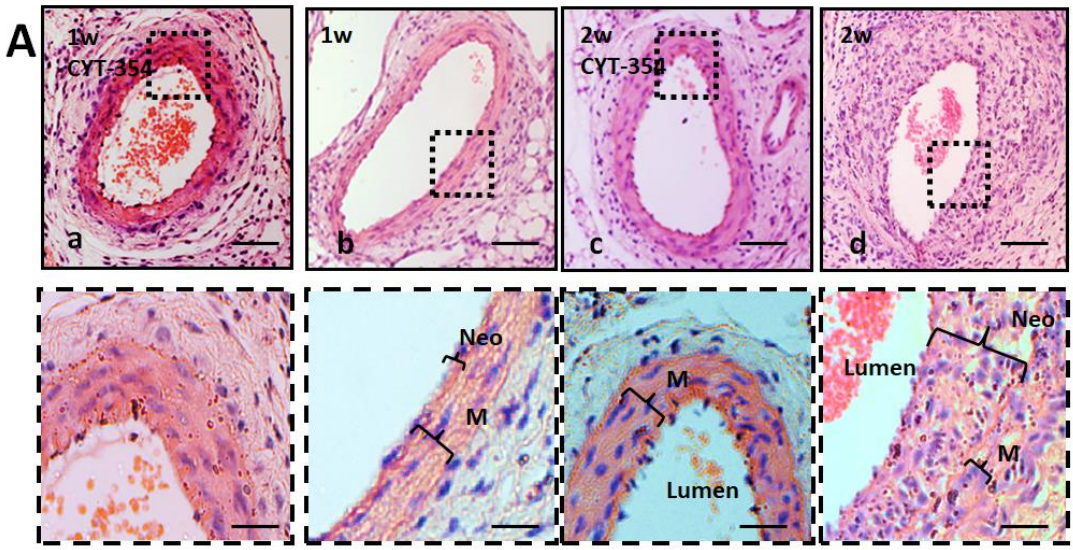


Figure 3.44. CYT-354 can significantly inhibit neointimal formation 2 weeks after endovascular injury. Sca-1^{+} progenitor cells (1*10^{6}) were seeded on the adventitia of previously injured vessels and allowed to migrate with or without CYT-354 in the Matrigel. **A**, Paraffin sections of injured femoral arteries were observed by performing H&E staining (scale bars, 50 and 10 μm, n=6). Dashed boxes represented the magnified field. Neo, neointima; M, media; 1W, 1 weeks; 2W, 2 weeks.

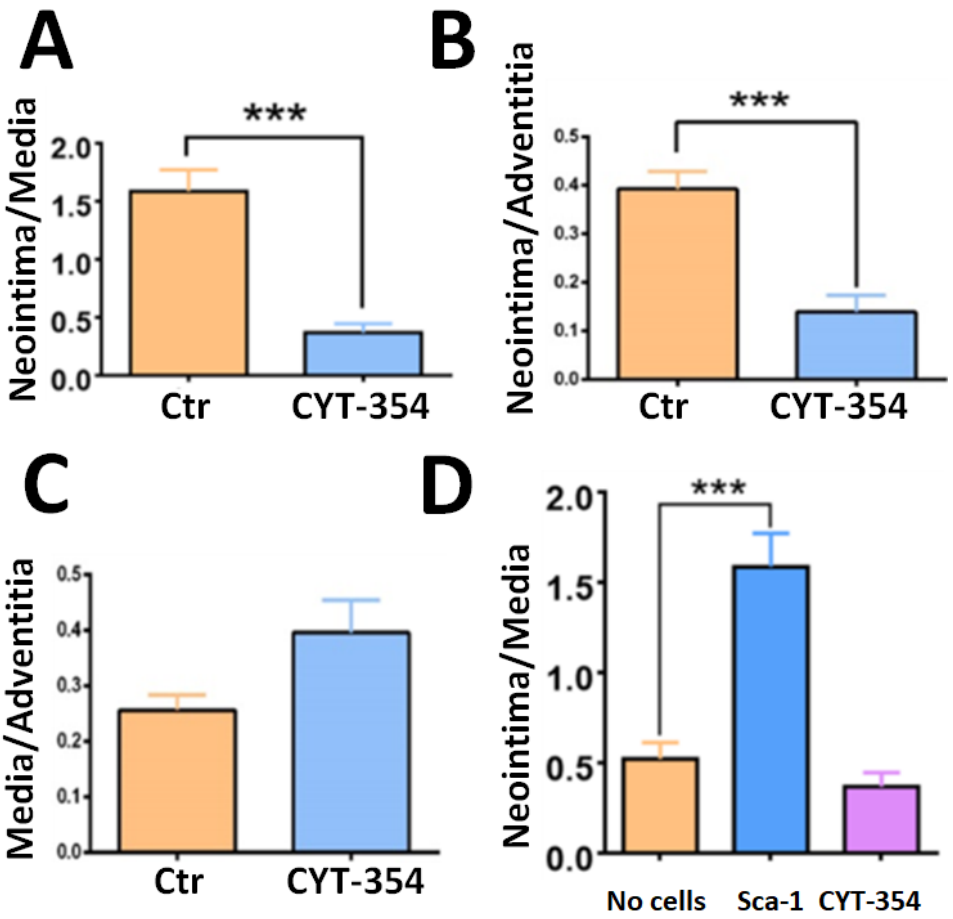


Figure 3.45. Transplantation of progenitor cells enhanced neointimal formation while CYT-354 significantly inhibited 2 weeks after endovascular injury. Sca-1^{+} progenitor cells (1*10^{6}) were seeded on the adventitia of previously injured vessels and allowed to migrate with or without CYT-354 in the Matrigel®. **A**, Quantification of ratio of neointima to media of injured arteries (n=6). **B**, Quantification of ratio of neointima to adventitia of injured arteries (n=6). **C,** Quantification of ratio of media to adventitia of injured arteries (n=6). **D**, Quantification of ratio of neointima to media of injured arteries with or without transplanted cells (n=6). Graphs are shown as mean ± SEM. Dashed boxes represented the magnified field. *P<0.05, **P<0.01, ***P<0.001. Neo, neointima; M, media; 1W, 1 weeks; 2W, 2 weeks.

3.12 Wire Injury Induces Neointimal Formation in Wild-type But Not in db/db Mice

We performed guide-wire injury of femoral arteries in db/db mice. The injured arteries were collected 2 or 4 weeks after surgery. The size of neointimal formation measured in db/db mice was much smaller than wild-type mice two weeks after the injury (Figure 3.47A). Of note, they had a higher concentration of leptin in the circulation (Figure 3.36), implying that the absence of leptin receptor may serve as a protective role in vascular remodeling. Previous reports have suggested that the lack of neointimal formation in db/db mice was mainly due to the inhibition of smooth muscle cells proliferation (Stephenson et al., 2003). We confirmed this conclusion by performing BrdU proliferation assays for SMC and Sca-1$^+$ adventitial progenitor cells from wild-type or db/db mice. Data showed that the absence of OBR could significantly decrease the proliferative abilities of both SMC and progenitors (Figure 3.46A and B). Taken together, db/db mice could not develop neointimal formation, which was probably caused by reduced ability of proliferation in smooth muscle cells and Sca-1$^+$ progenitor cells.

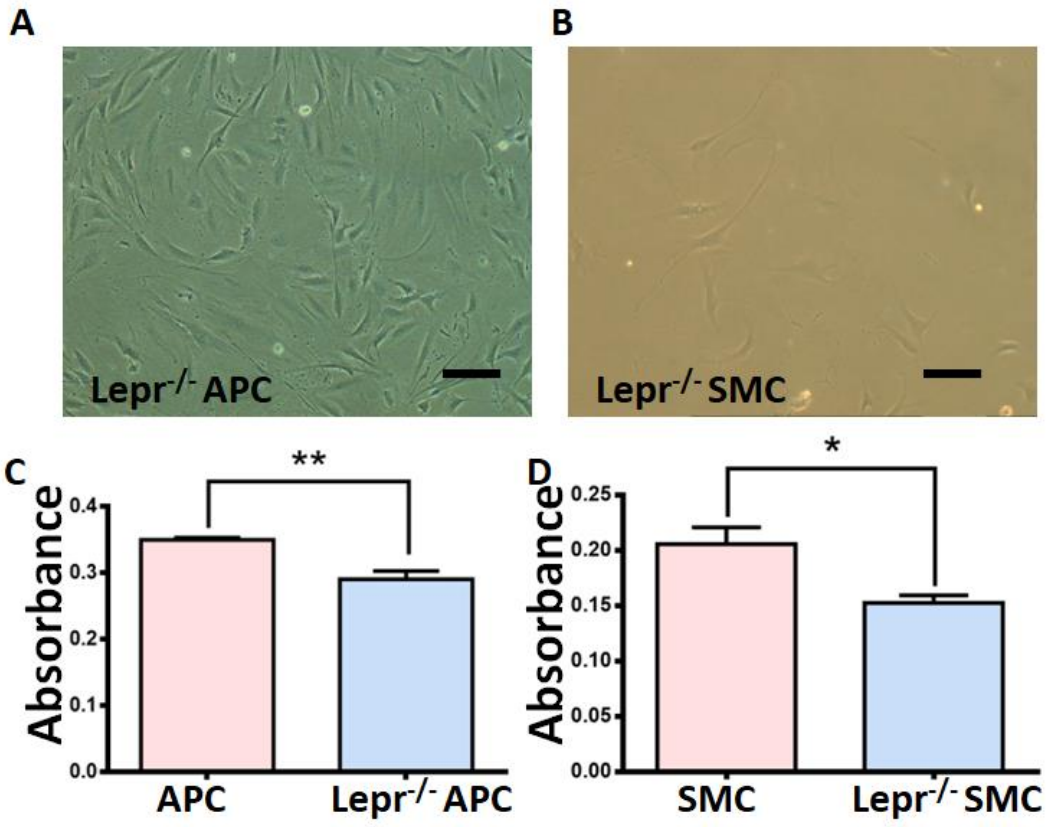


Figure 3.46. SMC from db/db mice showed a difficulty to expand *in vitro.* **A**, Morphology of Sca-1^{+} progenitor cells from db/db mice. **B**, SMC from db/db mice had difficulty to proliferate *in vitro*. **C** and **D**, Proliferation between Lepr$^{-/-}$ or lepr$^{+/+}$ Sca-1^{+} progenitors (C, n=4) and SMC (D, n=4) was examined by BrdU assay after 16-hour incubation with 100 ng/mL leptin. Serum-free cultured medium without leptin treatment was used as a control for the migration assays above. Figures shown above are representative of at least 3 separate experiments (scale bars, 50 μm). APC indicates Sca-1^{+} adventitial progenitor cells; SMC indicates smooth muscle cells. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

3.13 Neointimal Accumulation of Sca-1$^+$ Progenitor Cells Is Mediated by Leptin Receptor

To better understand the specific role of OBR expressed on Sca-1$^+$ progenitor cells in neointimal formation, the guide-wire injury mouse model was performed in wild-type and db/db mice and followed with transplantation of Sca-1$^+$ progenitor cells. Briefly, experiments were performed on two groups of animals: one group of wild-type mice underwent guide-wire injury, followed by a transplantation of $1*10^6$ Lepr$^{-/-}$ Sca-1$^+$ progenitor cells on the adventitial side of injured artery; another group of db/db mice underwent the same procedure but received $1*10^6$ Lepr$^{+/+}$ Sca-1$^+$ progenitor cells. The injured arteries were harvested 2 weeks after the surgery.

Our data revealed that in db/db mice receiving Lepr$^{+/+}$ Sca-1$^+$, there was a significant increase in neointimal formation (Figure 3.47B) compared to the group with injury only (Figure 3.47A and 3.48A). Lepr$^{-/-}$ Sca-1$^+$ cells (Figure 3.47D), on the contrary, could not enhance neointimal lesion in wild-type mice compared to the group with injury and the transplantation of Lepr$^{+/+}$ Sca-1$^+$ cells also in wild-type mice (Figure 3.47C and 3.48B). Using the immunofluorescent staining for SMCs and ECs markers, we analyzed the composition of Sca-1$^+$-induced neointimal formations (Figure 3.47). Interestingly, there was a thick layer of CD31$^+$ cells in db/db mice two weeks after the vessel injury, which may serve as an example of protective role of neointimal formation (Figure 3.47A). Taken together, our data showed that the expression of OBR on Sca-1$^+$ progenitor cells is crucial for the formation of neointima.

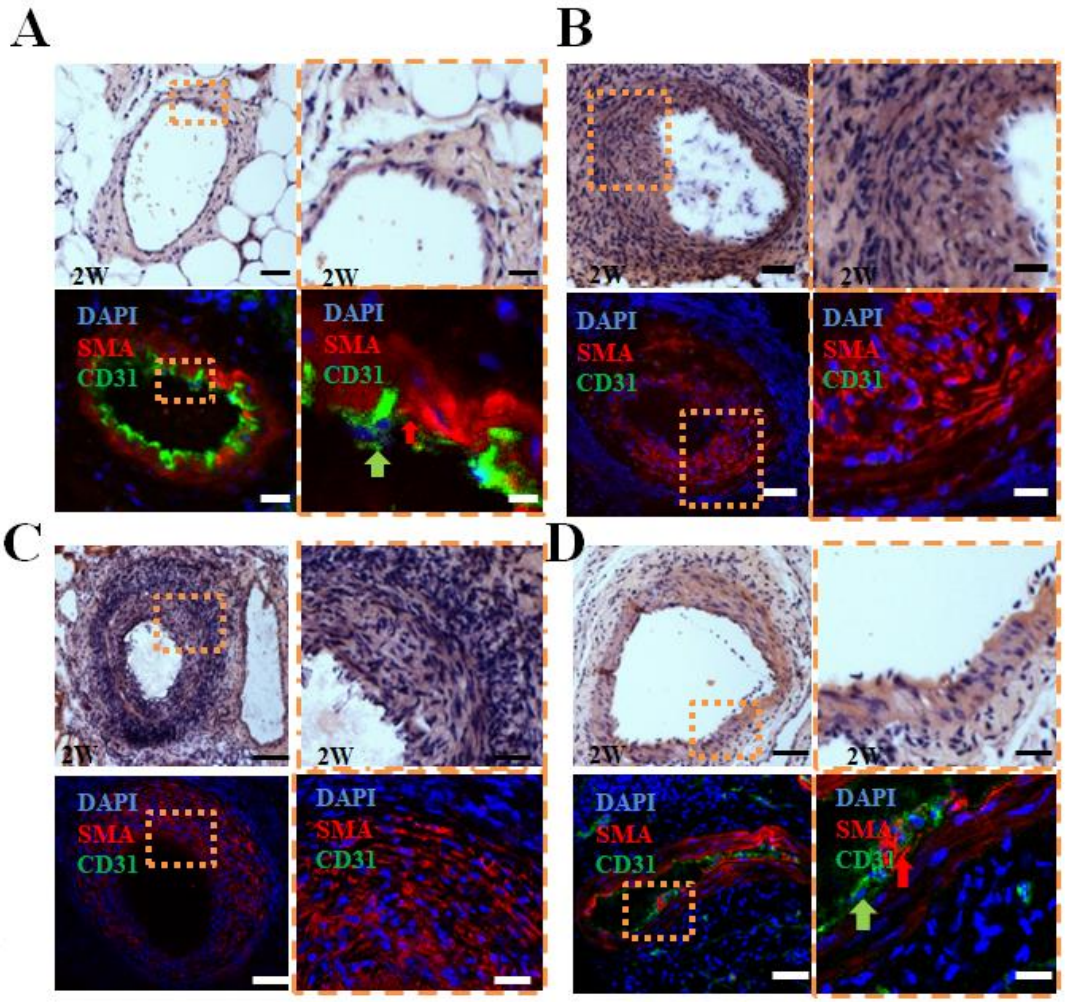


Figure 3.47. Neointimal Effect of Sca-1^{+} Progenitor Cells Is Mediated by Leptin Receptor. A, Cross sections of femoral artery from db/db mice at 2 weeks after the surgery were analyzed by H&E staining and immunofluorescence for αSMA and CD31 (scale bars, 50 and 10 μm, n=6). **B**, Lepr$^{+/+}$ Sca-1^{+} progenitor cells were seeded on the adventitial side of injured artery in db/db mice. Cross sections of femoral artery from db/db mice at 2 weeks after the surgery were analyzed by H&E staining and immunofluorescence for αSMA and CD31 (scale bars, 50 and 10 μm, n=5). **C**, Lepr$^{+/+}$ Sca-1^{+} progenitor cells were seeded on the adventitial side of injured artery in wild-type mice. Cross sections of femoral artery from wild-type mice at 2 weeks after the surgery were analyzed by H&E staining and immunofluorescence for αSMA and CD31 (scale bars, 50 and 10 μm, n=8). **D**, Lepr$^{-/-}$ Sca-1^{+} progenitor cells were seeded on the adventitial side of injured

artery in wild-type mice. Cross sections of femoral artery from db/db mice at 2 weeks after the surgery were analyzed by H&E staining and immunofluorescence for αSMA and CD31 (scale bars, 50 and 10 μm, n=6). Dashed box represented the magnified field. Arrows indicated the SMC and EC respectively. EC indicates endothelium; SMC, smooth muscle cells; Neo, neointima; M, media; SMA, smooth muscle actin; 2W, 2 weeks; 4W, 4 weeks. APC: adventitial progenitor cells.

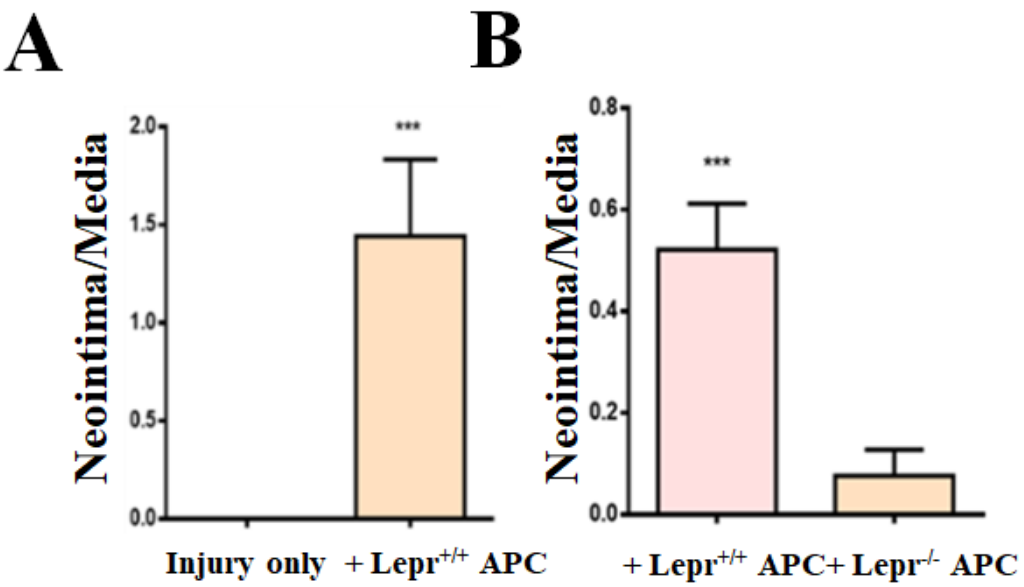


Figure 3.48. Leptin Receptor deficient APC could not induce neointimal formation 2 weeks post-surgery. A, Quantification of the ratio of media to adventitia of injured arteries for group A and B in figure 3.42. **B**, Quantification of the ratio of media to adventitia of injured arteries for group C and D in figure 3.42. Graphs are shown as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

3.14 Neointima in Db/Db Mice Partly Originated From Exogenous Sca-1^{+} Progenitors

When Lepr$^{+/+}$ Sca-1^{+} progenitors were transplanted to db/db mice, an apparent formation of neointima could be detected. Since db/db arteries were unable to develop any neointimal lesions following guide-wire injury only, the origin of neointima in the db/db mice following cell transplantation was of interest. Db/db mice do not express OBR in any cell types. Immunostaining did not show any positive expression of OBR in the femoral artery (Figure 3.9C). When Lepr$^{+/+}$ Sca-1^{+} cells were applied in db/db mice, this group of cells became the only origin of Lepr positive cells or tissues. Therefore, we performed immunostaining with OBR primary antibody in db/db mice which received exogenous Lepr$^{+/+}$ progenitor cells. Immunostaining of OBR revealed a significant number of Lepr^{+} cells in the neointima, indicating that they originated from transplanted Lepr$^{+/+}$ Sca-1^{+} progenitor cells (Figure 3.49A). It is worth noting that not all neointimal cells were OBR positive and most of the OBR positive cells did not co-express smooth muscle cell marker. Since db/db mice could not develop any neointimal formation after vessel injury, the origins of OBR negative cells and the roles of OBR positive cells in neointima in db/db mice will need further investigation. On the other hand, when Lepr$^{-/-}$ Sca-1^{+} cells were applied in wild-type mice, immunostaining revealed that OBR expressed in all three layers, which was considered as an intrinsic expression in wild-type mice, and neointima could not be increased (Figure 3.49B). In order to elucidate whether hematopoietic cells could contribute to the neoinitmal formation, we performed the immunostaining with CD45 as a hematopoietic marker and OBR represented as exogenous Sca-1^{+} progenitor cells primary antibody in db/db mice (Figure 3.50). Our data demonstrated that both adventitial and hematopoietic progenitor cells could contribute to neointima.

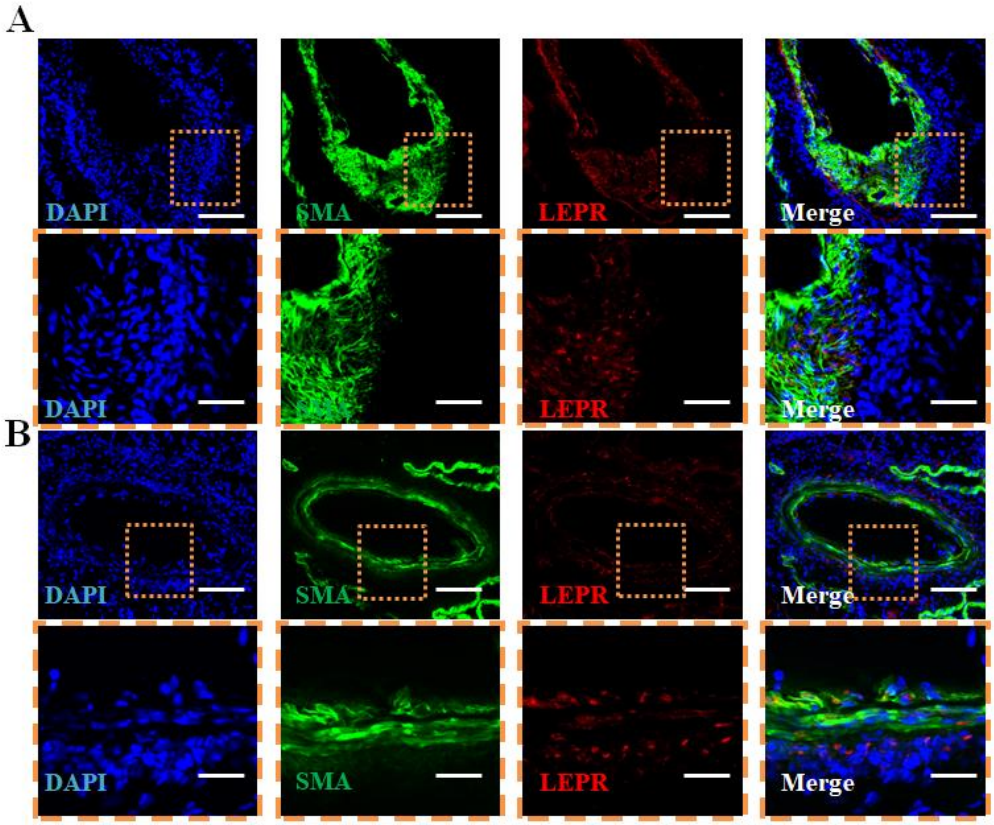


Figure 3.49. Neointima was originated from Lepr^{+} Sca-1^{+} Progenitor Cells. A, Lepr$^{+/+}$ Sca-1^{+} progenitor cells were seeded on the adventitial side of injured artery in db/db mice. Cross sections of femoral artery from db/db mice at 2 weeks after the surgery were analyzed by immunofluorescence for αSMA and OBR (scale bars, 50 and 10 μm, n=5). **B**, Lepr$^{-/-}$ Sca-1^{+} progenitor cells were seeded on the adventitial side of injured artery in wild-type mice. Cross sections of femoral artery from wild-type mice at 2 weeks after the surgery were analyzed by immunofluorescence for αSMA and OBR (scale bars, 50 and 10 μm, n=5). Dashed box represented the magnified field.

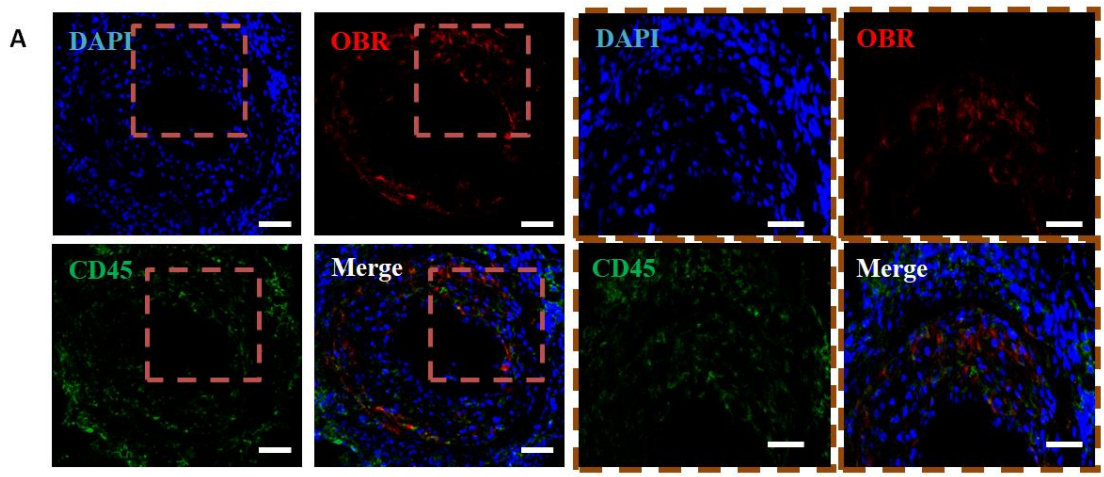


Figure 3.50. Both adventitial Sca-1^{+} cells and hematopoietic cells contribute to neointimal formation. A, Lepr$^{+/+}$ Sca-1^{+} progenitor cells were seeded on the adventitial side of injured artery in db/db mice. Cross sections of femoral artery from db/db mice at 2 weeks after the surgery were analyzed by immunofluorescence for CD45 and OBR (scale bars, 50 and 10μm, n=4). Dashed box represented the magnified field. Images shown are representative of at least 3 independent experiments.

Chapter 4
Discussion

Overview

Obesity is related to cardiovascular disease, but the underlying mechanisms behind obesity-induced cardiovascular disease are not elucidated. Plasma leptin, as an adipocyte product, is proposed to be a biological predictor of cardiovascular disease (Chu et al., 2001). The focus of atherosclerosis is traditionally on endothelium which is the innermost layer of vessels. The deeper layers including media, adventitia and perivascular adipose tissue, which were proved to be crucial for vascular remodeling, received far less attention. Accumulating evidence indicates the presence of vascular progenitor cells in all three layers of large and medium-sized blood vessels, and these progenitors may participate in vascular remodeling (Cho et al., 2013; Naito, Kidoya, Sakimoto, Wakabayashi, & Takakura, 2012; Patel et al., 2017; Z. Tang et al., 2012; Worsdorfer et al., 2017). Recent studies linked adipokines and vascular smooth muscle cells. Heart and vascular smooth muscle cells are capable of secreting leptin (Hou & Luo, 2011), which can subsequently enhance coronary vasoconstriction and smooth muscle proliferation *via* the Rho kinase pathway (Noblet, Goodwill, Sassoon, Kiel, & Tune, 2016). What is more, the hypothalamus resistance to leptin did not affect the heart and vascular walls (Martin, Qasim, & Reilly, 2008). All these findings revealed a possible connection between adipokines, vascular progenitor cells and cardiovascular system. However, the relationship between adventitial progenitor cells and adipokines remained until now unknown.

In the present study, we have identified the missing link between leptin and the resident stem cells in vascular remodeling. We demonstrated that leptin was a chemokine for stem/progenitor cell migration and mediated neointimal formation in damaged vessels, which can be abrogated by inhibition or knockout of the leptin receptor. Modulation of the signaling pathways involving OBR-STAT3-Rac/Cdc42-FAK influences cell motility *in vitro* and

vascular remodeling in vessel-injury models. More importantly, for the first time, we have successfully shown significant neointimal formation in db/db mice, confirming the crucial roles of leptin receptor b (OBRb) and Sca-1^{+} progenitors in vascular remodeling. Thus, our findings provide a novel insight into the interaction between leptin and vascular resident progenitor cells in the pathogenesis of vascular disease.

4.1 Characterization of Sca-1$^+$ Progenitor Cells

Mature arteries are mainly composed of by two cell types, smooth muscle cells and endothelial cells (Xiong, 2008). It is well known that both endothelial cells and smooth muscle cells can contribute to the development of atherosclerosis. The proliferation of smooth muscle cells in the neointima is considered as the leading cause of atherosclerosis but their origin is still under debate. It was initially believed that smooth muscle cells come from the media in response to the chemokines such as PDGF and TGF-β released by endothelial cells or aggregated platelets (Russell Ross, Glomset, & Harker, 1977). However, increasing evidences suggested that vascular progenitor cells from adventitia can contribute to atherosclerosis as well.

Far less attention has been paid to the adventitia of vessel wall. The adventitia used to be only regarded as the outermost connective layer of the artery but recent studies revealed more roles of the adventitia in vascular remodeling. The adventitial layer contains heterogeneous groups of cells. Besides resident macrophages, mast cells, T cells, B cells, and dendritic cells were also discovered in the adventitia (Galkina et al., 2006; Mäyränpää et al., 2009; J. Zhou et al., 2010). Also, adventitia is proved to contain progenitor cells which can participate in atherosclerosis and other pathological processes (Campagnolo et al., 2010; Hoshino et al., 2008; Passman et al., 2008). Vascular adventitial progenitor cells can differentiate into either endothelial cells or smooth muscle cells in certain circumstance. For instance, CD34$^+$ vascular progenitor cells derived from embryonic stem cells could differentiate into CD31, VE-cadherin positive endothelial cells under VEGF stimulation and sm22α, or calponin positive smooth muscle cells under PDGF-β stimulation (Ferreira et al., 2007). Sca-1$^+$ cells are non-bone marrow derived vascular progenitor cells, found abundantly in vessel walls and subsequently cultured in stem cell medium which were also capable of differentiating into endothelial cells, smooth muscle cells and cardiomyocytes

(Czechanski et al., 2014; Y. Hu et al., 2004; Qingzhong Xiao, Zeng, Zhang, Hu, & Xu, 2007). Using an animal model of vein graft, Hu et al. proved that about 60% of the smooth muscle cells were from the donor's vessel wall, and about 40% of the smooth muscle cells were from the recipient, possibly from blood (Y. Hu et al., 2002). In another experiment, Sca-1^{+} progenitor cells carrying LacZ reporter gene were isolated from the aortic root of apoE$^{-/-}$ mice. The vena cava was transplanted into the carotid artery of the recipient mice, while the donor Sca-1^{+} Lac^{+} cells were applied on the adventitial side of the carotid artery. Once atherosclerotic lesions formed, LacZ-smooth muscle cells were observed in the grafts, suggesting the migration and differentiation of the Sca-1^{+} progenitor cell from adventitia (Y. Hu et al., 2004).

Besides, vessels, Sca-1^{+} progenitor cells also exist in other tissues, such as bone marrow and heart, and they have different roles from region to region. Sca-1^{+} was reported as a marker on hematopoietic progenitor cells, in combination with c-kit. Also, Sca-1^{+} markers were identified in the resident fibroblast of skin (Driskell et al., 2013), lung (Akamatsu et al., 2013), which could differentiate into hypodermal lineage and myofibroblasts respectively. Resident cardiac progenitor cells are a promising treatment for myocardial infarction. Heart derived Sca-1+ progenitor cells showed the characteristics of cardiac progenitor cells, demonstrating multipotent differentiation into cardiac cell lineages (H. Wang et al., 2014).

In this study, we isolated Sca-1^{+} progenitor cells from the aortic of apoE$^{-/-}$ (Lepr$^{+/+}$) mice and db/db (Lepr$^{-/-}$) mice. This group of cells physically located in the adventitial of big vessels and will undergo a series of cell activities under pathological conditions. Application of Sca-1^{+} progenitor cells to the wire-injured femoral artery allowed us to create a reasonable representation of vascular dysfunction in a relatively short time. Mice were chosen for animal surgery because of its fast breeding and easy access to gene deficient models compared to other big animals such as rabbits and rats. By performing such experiments, we believed that our findings will be instructive

for future studies in vascular remodeling.

4.2 Leptin, OBR and Sca-1$^+$ Progenitor Cells

Obesity strongly correlated with higher concentration of serum leptin associated with higher incidence of cardiovascular diseases. Leptin is a 16 kDa peptide hormone mainly secreted by white adipose tissue. Leptin, which was firstly discovered in 1994, was initially considered as a regulator of metabolism. However, accumulating evidence revealed that leptin also played an important role in cardiovascular system.

Schafer et al. confirmed that wild-type mice would have 9-fold higher leptin levels in the circulation with high fat diet. The lesions of neointima in carotid artery were also strongly enhanced in mice with high-fat diet. Moreover, leptin deficient (ob/ob) mice could not develop neointimal formation spontaneously, but the neointimal formation in ob/ob mice could be developed with the treatment of exogenous leptin (Schäfer et al., 2004). Leptin could induce the proliferation and migration of vascular smooth muscle cells and neointimal formation, the phenomenon of which could be suppressed genistein (Y. C. Tsai et al., 2017). In addition, leptin regulated the osteoblastic differentiation of vascular smooth muscle cells by increasing the expression of RANKL (Liu et al., 2014). A growing body of evidence showed the effects of leptin on vascular smooth muscle cells, endothelial cells and progenitor cells. However, little attention has been drawn to adventitial progenitor cells.

Obesity leads to changes in the levels of plasma adipokines such as adiponectin, leptin, visfatin, and resistin, all of which may influence cardiovascular system. To determine whether Sca-1$^+$ progenitors could response to these adipokines, migratory assays including transwell (Figure 3.1A) and wound-healing (Figure 3.1B) in response to different adipokines were performed. We confirmed that only resistin and leptin could induce the migration of Sca-1$^+$ progenitor cells. On the contrary, adiponectin inhibited the

migration of Sca-1$^+$ progenitor cells. Since the migration of Sca-1$^+$ could enhance the neointimal formation in previous study, we inferred that leptin and resistin could probably play a role in vascular remodeling. It is clear that OBRb is considered to be the functional receptor which mediates most of the biological leptin-induced effects (Yang & Barouch, 2007). However, the receptors of resistin were still under debate. OBR is expressed in multiple cell types (Koh, Park, & Quon, 2008). Single cell transcriptome analysis from previous work in our lab demonstrated that Sca-1$^+$ progenitor cells express long-form leptin receptor (data not shown, in press), followed by confirmation with Western blotting and immunostaining experiments (Figure 3.6, 3.7 and 3.9). Therefore, we chose leptin as our main focus in this project, exploring the relationship between leptin, Sca-1$^+$ progenitor cells and neointimal formation.

4.3 Db/Db Mice, Type II Diabetic Model and Vascular Injury

Db/db mice are greatly applied as a type II diabetes model. Db/db mice fed a Western diet shows a similar characteristics to that in type II diabetes mellitus(DM) patients, with higher expression of low-density lipoprotein (LDL) and cholesterol (Kobayashi et al., 2000), both of which can enhance the neointimal formation. Diabetic

Dyslipidemia greatly contributes to the accelerated atherosclerosis in Diabetic mellitus. In previous study, guide-wire injury mouse model was performed in both type I and II diabetes mellitus mouse models, creating neointima which could be accelerated by arterial injury. In rodent model of type I diabetes, neointimal size was not altered after the induction of guide-wire injury of femoral artery in ins2^{akita} mice and Sprague-Dawley rat treated with streptozotocin (Park et al., 2001). Surprisingly, in db/db mice, neointimal formation was greatly reduced even with the help of upregulation

of serum leptin, cholesterol and LDL. Leptin can increase sympathetic nerve activity and arterial blood pressure (Aizawa-Abe et al., 2000). Arterial rings harvested from ob/ob and db/db mice could response to norepinephrine and diminish relaxation to acetylcholine, enhancing contractile activity. However, increased mechanical force of injured artery did not explain the diminished neointima in db/db mice after vessel injury (Winters et al., 2000). TUNEL-positive staining 4 hours post-surgery and Ki67 staining 2 weeks post-surgery was unchanged compared to $Lepr^{+/+}$ mice. Therefore, apoptosis or proliferation of smooth muscle cells in the medial layer of db/db mice did not account for the paradoxical reduction of neointimal formation (Stephenson et al., 2003). Since leptin has a wide range of effects on vascular cells including platelet and VSMC, it is difficult to figure out the paradox without additional specific mutant mouse model. In summary, this paradoxical effect demonstrates a potential role of leptin receptor b in vascular remodeling as a protective role.

4.4 Leptin Induce Sca-1$^+$ Progenitor Cells *via* OBR

Adventitial progenitor cells from aortic adventitia could contribute to neointimal formation. These cells express progenitor cell markers, such as Sca-1, CD34 and CD117, and are localized in neointima (T.-N. Tsai et al., 2012) and adventitia (Ergün, Tilki, & Klein, 2011). Several studies has revealed that the migration of Sca-1$^+$ progenitor cells can be induced by a wide range of stimuli, such as Sirolimus (Mei Mei Wong et al., 2013a), and participate in atherosclerosis. Since the expression of leptin receptor b (OBR) in Sca-1$^+$ progenitor cells was confirmed as mentioned previously, we wondered whether leptin could induce the migration of Sca-1$^+$ progenitor cells *via* OBR.

We firstly isolated the Sca-1$^+$ adventitial progenitor cells from both wild-type and db/db mice. To investigate whether the absence of OBR would affect the

phenotype or distribution of Sca-1$^+$ progenitor cells *in vitro* and *in vivo*, we performed a series of immunostaining for both cells and tissue. Our data revealed that there was no any expression of OBR in db/db mice, and the distribution of Sca-1$^+$ progenitor cells was not changed in comparison to the distribution in wild-type mice (Figure 3.9 B and C). FACS results suggested that Sca-1$^+$ progenitor cells expressed CD29, CD34 but not CD45. The absence of OBR did not change the phenotype of Sca-1$^+$ progenitors. In summary, the expression of OBR is independent with the expression of Sca-1 and other progenitor markers.

Migratory assays including transwell and wound healing assays were performed. Cell migration could be significantly enhanced in response to 100 ng/mL of leptin in both transwell assay and wound healing assay. The leptin-induced migration could be abolished in response to Leptin antagonists which competitively bound to OBR. Leptin antagonist triple mutant mouse recombinant used in this project is a single non-glycosilated polypeptide chain, with alanine mutagenesis of three amino acids (L39A/D40A/F41A), resulting in potent antagonistic activity. In addition, applying Lepr$^{-/-}$ Sca-1$^+$ progenitor cells in both transwell and wound-healing assays demonstrated a reduced migration. Treatment with different concentrations of leptin in Lepr$^{-/-}$ Sca-1$^+$ progenitor cells did not show any difference regarding the cell migration. Above data suggested that leptin induced the migration of Sca-1$^+$ progenitor cells *via* OBR.

4.5 The Underlying Mechanism of Leptin-Induce Cell Migration of Sca-1$^+$ Progenitor Cells

Leptin receptor codes for six leptin receptor isoforms *via* RNA splicing. All of them have an extracellular domain which can bind to leptin but only leptin receptor b (OBR) contains a full-length intracellular domain which is necessary for signal transduction cascade (Friedman & Halaas, 1998). The

action of OBR is commonly induced by JAK/STAT (Zabeau et al., 2003), AMPK (Minokoshi et al., 2002), PI3-Akt and MAPK (Frühbeck, 2006) pathways.

Consistent with previous studies, leptin could induce the activation of phosphorylated STAT3, MEK1/2, and ERK1/2 at an early stage. Either inhibition of OBR, STAT3 or ERK1/2 led to a reduction in cell migration *in vitro*. However, a late activation of pERK at 30 minutes after the treatment in $Lepr^{-/-}$ cells indicated that other signaling pathways independent of OBR may also be involved. We hence performed a qPCR for the expression of cell migration-related receptors such as CCR 1, 2, 7, 9 and CXCR 3, 4, 5 as it was shown previously that they were upregulated in Sca-1^{+} progenitor cells during migration (Mei Mei Wong et al., 2013b). Surprisingly, the expression of CXCR5 gene (Figure 3.19) was upregulated 24 hours after the treatment of leptin, indicating its potential role in leptin-induced signaling pathways. Previous study demonstrated that the activation of STAT3 in follicular helper T (TFH) cells could directly induce the expression of CXCR5 (Tripathi et al., 2017). Also, Overexpression of CXCR5 in mesenchymal stem cells increased their abilities to respond to migratory stimuli and immunomodulatory effects *in vivo* (X. Zhang et al., 2017). However, whether the activation of STAT3 in Sca-1^{+} progenitor cells could enhance the expression of CXCR5 cannot be concluded. We also performed MAPK protein arrays on leptin-stimulated Sca-1^{+} progenitors. Various phosphorylated MAPK proteins were activated (Figure 3.20). CXCR5 has never been reported as a receptor of leptin. Nevertheless other MAPK proteins were related to leptin-induced signaling pathway. Thus, an interaction between CXCR5, MAPK pathway and leptin still needs further investigation. The Rho GTPase family including Rac1, Cdc42, and RhoA is known to regulate the formation of lamellipodia, filopodia and focal adhesions (Nobes & Hall, 1999). In our study, Rac1 and Cdc42, commonly considered to act upstream of MAPK pathways, were activated by 100 ng/mL of leptin, followed by an enhanced rearrangement of

cytoskeleton-related proteins such as phosphorylated FAK and vinculin.

Vinculin and FAK are believed to have a close relationship with cell migration. Previous work from our laboratory proved that vinculin and FAK can be activated in Sca-1$^+$ progenitor cells by a wide range of stimuli, eventually leading to cell migration (Yu et al., 2016). However, the influence of leptin on vinculin and FAK in Sca-1$^+$ progenitor cells was so far unclear. Our new data showed that leptin potently greatly induced vinculin and FAK activation in 30 minutes in stem/progenitor cells, and it strongly correlated with the phosphorylation of STAT3-GTPase-ERK pathway. Taken together, we provided robust data to identify OBR-STAT3- Rac1/Cdc42-ERK-FAK as most probable signaling pathway involved in Sca-1$^+$ progenitor cell migration in response to leptin, implicating new potential therapeutic targets for vascular disease. Schematic figure for leptin-induced signaling pathway in Sca-1$^+$ progenitor cell migration was displayed on Figure 4.1.

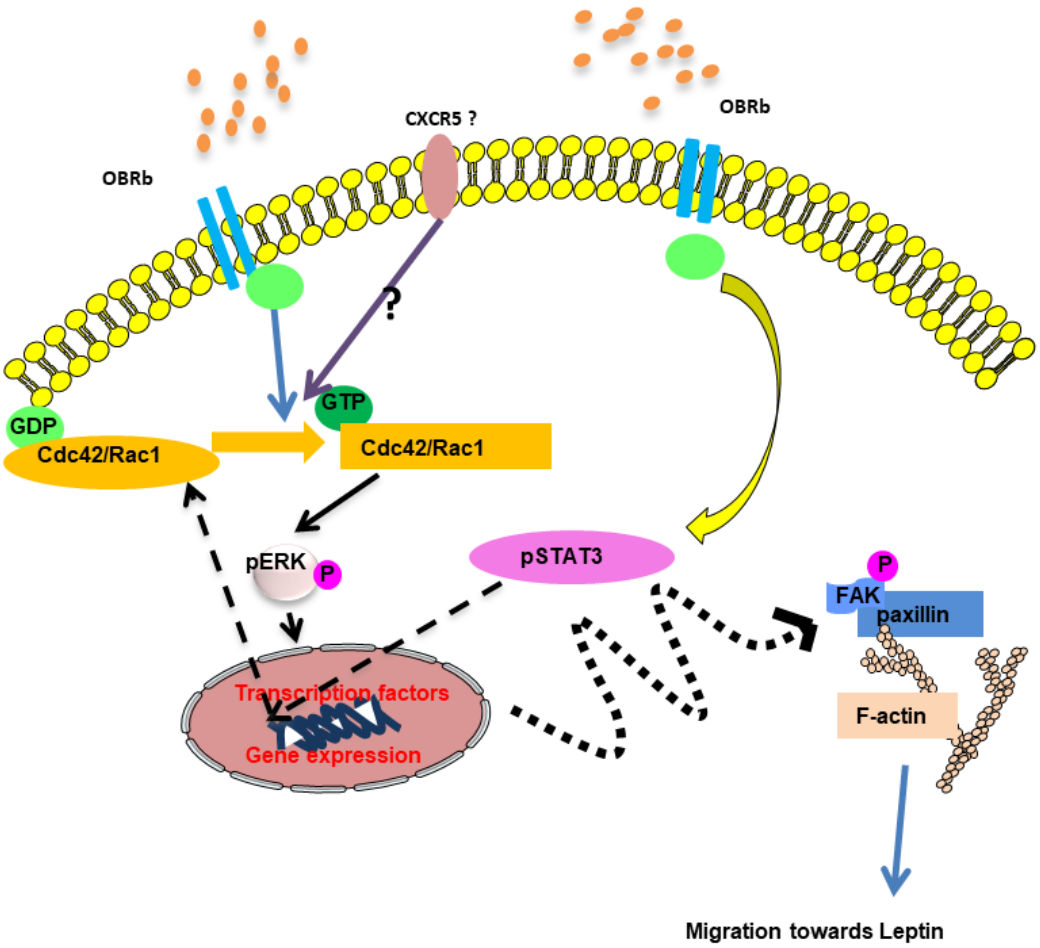


Figure 4.1. Schematic illustration of the roles of exogenous leptin in enhancing Sca-1$^+$ adventitial progenitor cells chemotaxis. Elevated leptin in circulation or femoral artery after guide-wire injury binds to its receptors leptin receptor b on the Sca-1$^+$ adventitial progenitor cells. The GTPases Rac1 and Cdc42 are activated leading to the phosphorylation of pERK. Leptin also induces the activation of STAT3 pathway and the expression of cytoskeleton related proteins paxillin, vinculin and phosphorylated FAK, which may eventually contribute to cell migration.

4.6 Guide-Wire Injury Enhanced the Expression of Leptin Locally and Systematically, Inducing Sca-1$^+$ Progenitor Cells Migration.

Atherosclerosis is a chronic cardiovascular disease which can be asymptomatic for decades. It is also the leading cause of stroke, myocardial infarctions and other vascular diseases of which mechanism is still not fully understood. Various animal models are hence developed (Getz & Reardon, 2012). In the present study, we used guide-wire injury mouse model due to its accessible manipulation and relatively quick outcome.

First applied by Roque et al. in 2000, guide-wire injury model soon became widely accepted as it closely resembled the injury of the endothelium and vessel wall. Guide-wire injury to the vessel can be achieved by 3 passages of a 0.25mm diameter angioplasty guide wire. One hour after the injury, the denuded surface was covered with platelets and leukocytes due to the accumulation of P-selectin, intercellular adhesion molecule-1 and vascular cell adhesion molecular-1 within the injured area. One week post-surgery, macrophages and platelets were observed in the vessel wall. Two or four weeks post-surgery, guide-wire injury induced a significant neointimal hyperplasia along with the rapid accumulation of leukocytes and adhesion molecules on the regenerated endothelium (Roque et al., 2000).

After the endovascular injury, an inflammatory response take place in the vessel wall, with the release of chemokines secreted by smooth muscle cells and mononuclear cells (Tedgui & Mallat, 2006). However, whether the expression of leptin was affected was so far unknown. We demonstrated that the levels of leptin in both serum and vessel wall were upregulated one day post-surgery. Since higher concentration of leptin could induce the cell migration *in vitro*, we believed that the upregulation of leptin *in vivo* could also induce the migration of Sca-1$^+$ progenitor cells from the adventitia to the intima. Although leptin was reported to be mainly released by white adipose

tissue (Trayhurn & Beattie, 2001), our immunostaining showed that smooth muscle cells could also express leptin both *in vivo* and *in vitro*, providing a new possibility of additional origin of leptin after the endothelial injury.

Using a RFP labelling/tracing technique, migratory Sca-1$^+$ cells could be traced inside the vessel media layer. Lepr$^{-/-}$ cells or Lepr$^{+/+}$ treated with CYT-354 lost their migratory abilities both *in vitro* and *in vivo*. However, when Lepr$^{+/+}$Sca-1$^+$ progenitor cells were transplanted on injured arteries of db/db mice which although had a much higher concentration of serum leptin, the migration of the progenitor cells was not significantly altered. On the other hand, Lack of OBR in db/db mice did not affect the migration of Lepr$^{+/+}$ Sca-1$^+$ progenitor cells. Therefore, progenitor cell migration *in vivo* is largely dependent on the existence of leptin receptors on the cell surface. Moreover, Lepr$^{+/+}$Sca-1$^+$ progenitor cells could acquire smooth muscle cell markers *in vivo* 3 days post-surgery. Quantitative RT-PCR in Sca-1$^+$ cells showed an upregulation of SMC markers in response to 100 ng/mL of leptin for 24 hours, suggesting that leptin may have a potential role in the differentiation of the progenitors towards the SMC fate *in vivo* (Figure 3.33). Further investigation will be needed to confirm a link between leptin and Sca-1$^+$ progenitor cells' differentiation.

4.7 Leptin Induced Neointimal Formation, Which Was Originated From Sca-1$^+$ Progenitor Cells.

The origin of the proliferative SMCs accumulating during neointimal formation is still under debate. Fibroblasts (Sartore et al., 2001), smooth muscle cells and adventitial progenitor cells (Torsney, Hu, & Xu, 2005) are all believed to participate in neointimal formation. In this context, leptin has been reported so far to promote integrin-mediated adhesion, recruiting endothelial progenitor cells into neointima after vessel injury (Schroeter et al., 2008). Db/db mice displayed phenotype of obesity, higher systemic arterial blood pressure,

depressed heart rates, hyperlipidemia, severe hyperglycemia, hyperinsulinemia and pancreatic dysfunction (da Costa Goncalves et al., 2009; B. Wang, Charukeshi Chandrasekera, & J Pippin, 2014), all of which could contribute to vascular remodeling.

At first, we confirmed that db/db mice had a higher serum leptin. Surprisingly, no neointimal formation could be observed in db/db mice at 2 or 4 weeks post-operation of guide-wire injury. Besides, there was a thick layer of $CD31^+$ endothelial cells in the intima at 2 weeks post-surgery. Furthermore, application of $Lepr^{+/+}Sca\text{-}1^+$ progenitors on the adventitial side of the injured artery in db/db mice resulted in marked neointimal formation 2 weeks post-surgery. Previous reports have suggested that the lack of neointimal formation in db/db mice was mainly due to the inhibition of proliferation of smooth muscle cells (Stephenson et al., 2003). We confirmed this conclusion by performing BrdU proliferation assays for SMC and progenitor cells with or without OBR. Our data showed that the absence of OBR could significantly decrease the proliferative abilities of both SMC and progenitors (Figure 3.46), but stimulation with leptin does not influence the proliferation of wild-type cells (Figure 3.5).

Importantly, we also demonstrated that the inhibition of migration of $Sca\text{-}1^+$ progenitor cells is of great importance for neointimal formation. Without the participation of $Lepr^{-/-}$ smooth muscle cells or other circulating $Lepr^{-/-}$ progenitors in db/db mice, a significant neointimal formation was also detected 2 weeks post-surgery in db/db mice, solely caused by the transplantation of exogenous $Sca\text{-}1^+$ progenitor cells.

Due to the fluorescence quenching of RFP cells or loss of RFP marker after cell migration and differentiation *in vivo*, we are unable to track the cells *in vivo* two weeks post-surgery. Instead, we stained the cross section of db/db mice with OBR primary antibody which underwent the transplantation of $lepr^{+/+}$ $Sca\text{-}1^+$ cells. Surprisingly, many neointimal cells were OBR positive, proving that they originated from the transplanted $Sca\text{-}1^+$ progenitors. Since

OBR$^+$ cells only represented less than 20% of the neointimal cells, most of which did not co-express SMC marker, the origin of the remaining cells is unknown and needs further investigation. In addition, whether exogenous Lepr$^{+/+}$ progenitor cells could influence the biological feature of Lepr$^{-/-}$ cells in db/db remained unclear. On the contrary, wild-type mice which received Lepr$^{-/-}$ Sca-1$^+$ cells only showed few OBR$^+$ cells and smaller neointima. A progenitor specific knock out of OBR could be a therapeutic approach for vascular disease in the future. Due to technical limitations, we are not able to track all RFP Sca-1$^+$ progenitors *in vivo* in a long-term experiment. A future study using Cre-controlled cell linear tracing techniques will be needed for long-term tracing experiments and study the differentiation of the prognitors.

4.8 Future Work

4.8.1 The Role of Leptin in Sca-1$^+$ Progenitor Cells Differentiation.

In the present study, we demonstrated that leptin could induce Sca-1$^+$ progenitor cells migration both *in vivo* and *in vivo*, enhancing the neointimal formation in wire-injured femoral arteries. Previous studies revealed that Sca-1$^+$ progenitor cells could differentiate into smooth muscle cells during the vascular remodeling (Y. Hu et al., 2004). In addition, leptin promoted the osteoblastic differentiation towards smooth muscle cells (Liu et al., 2014). Therefore, we assessed whether leptin could induce the differentiation of Sca-1$^+$ progenitor cells. Our preliminary results obtained *in vitro* with qPCR and *in vivo* with immunostaining suggested that Sca-1$^+$ progenitor cells could acquire smooth muscle cells marker in response to high concentration of leptin. In this case, it would be of great interest to further explore the role of leptin in cell differentiation and its underlying mechanism.

4.8.2 Potential Interaction between Leptin and OBR-Independent Signaling Pathways.

We proved that OBR played a substantial role in Sca-1$^+$ progenitor cells migration. Lack or inhibition of OBR significantly abolished the cell migration both *in vivo* and *in vitro*. However, Western blotting revealed that there was a late ERK activation even in cells treated with STAT3 inhibitors or using Lepr$^{-/-}$ Sca-1$^+$ progenitor cells, suggesting that other OBR-independent signaling pathways might have been involved. QPCR of CXCR5 showed a marked increase in response to 100 ng/mL of leptin. Besides the activation of ERK1/2 and MEK1/2, MAPK array demonstrated that various MAPK signaling pathways were activated in response to 100 ng/mL of leptin at a very short period. Altogether, our data indicated that OBR-STAT3-ERK1/2 was the not only pathway, which could be activated by leptin. However, whether other receptors and signaling pathways are related to leptin-induced cell migration remains unknown. The inhibitor of such signaling pathways and receptor should be applied for the further investigation in the future experiments. Transcriptome analysis and CHIP assays for Sca-1$^+$ progenitor cells with or without leptin treatment could be performed for the selection of the most probable receptors and pathways that contribute to the effect which we observed. So far OBR was the only reported receptor for leptin, a novel receptor for leptin could be of great interest for the studies in the cardiovascular and metabolic research field.

4.8.3 Sca-1$^+$ Progenitor Cells Contribute to Vascular Remodeling.

Present study displayed that Sca-1$^+$ progenitor cells could migrate into intima 3 days post-surgery. However, this *in vivo* migratory model was achieved by the transplantation of large quantity of Sca-1$^+$ progenitor cells in Matrigel

outside the vessel wall. Autologous Sca-1$^+$ progenitor cells in wild-type mice could be detected using immunostaining for large vessels. However, upon injury, they are difficult to track due to limited numbers and loss of Sca-1 antigen. Thanks to the development of CreERT-technology, the Sca-1-Cre-RFP mice would allow us to study not only the migration of Sca-1$^+$ cells but also the destiny of the cells that originated from them. In this case, we would be able to trace the autologous Sca-1$^+$ progenitor cells for a relatively long time. Although we stained OBR in db/db mice as an alternative approach to trace the Lepr$^{+/+}$Sca-1$^+$ progenitor cells, after all it is not the direct evidence. Once the Sca-1-CreERT-RFP model is created, various experiments can be designed and proceeded subsequently, exploring both the differentiation and migration of Sca-1$^+$ progenitor cells *in vivo*.

4.8.4 The Relationship Between Adipokine, Adipose Tissue, Adipose Tissue Derived Stem Cell (ADSC) and Vascular remodeling

Adipose tissue, expanding during obesity, strongly correlated with cardiovascular diseases. Perivascular adipose tissue, the adipose surrounding the large vessels as a part of adventitia, was found to be a potentially cardiovascular risk which contributed to neointimal hyperplasia in humans (Manka et al., 2014; Tian et al., 2013). A new consensus is emerging that perivascular adipose tissue is associated with cardiovascular disease but direct proof is still lacking. In the present study, we demonstrated that leptin could induce the Sca-1$^+$ progenitor cells migration, which further enhanced neointimal formation. We also discovered that serum leptin was greatly upregulated one day post-surgery. However, the origin of leptin remained unknown. Adipokines including leptin could be released by adipose tissue, but the interaction between leptin, adipose tissue and neointimal formation remains unclear. Several studies have already examined the impact of

transplanted adipose tissue on local vasculature. In one study, endogenous adipose tissue was removed and replaced by either subcutaneous or visceral adipose tissue, followed by a guide-wire femoral artery injury (Takaoka et al., 2009). Subcutaneous fat from normal diet mice could inhibit neointimal formation but subcutaneous fat from high-fat diet fed mice had no such effect. In addition, visceral epididymal adipose tissue also showed diminished neointimal hyperplasia (Takaoka et al., 2009). However, in another article, atherosclerosis was quantified after transplantation of visceral or subcutaneous fat to the carotid artery and researchers found visceral epididymal adipose tissue could augment atherosclerosis whereas subcutaneous adipose tissue served as a protective role (Öhman et al., 2011). Above contradictive results from different groups suggested the complex roles of adipose tissue in vascular remodeling.

Adipose derived stem cells, or ADSCs, are a population of pluripotent cells which are located in adipose tissue (Zuk et al., 2002) and can differentiate into various cell lineages, such as adipocytes, osteocytes and neurons, etc (Gimble, Katz, & Bunnell, 2007). Several articles addressed its new roles in vascular remodeling (Y. J. Kim et al., 2007; Suzuki, Fujita, Takahashi, Oba, & Nishimatsu, 2015). We next plan to perform a series of animal models related to the transplantation of ADSC or adipose tissue, followed by guide-wire injury of femoral artery. A fully understood relationship between adipokine, adipose tissue, ADSC and vascular remodeling would be of great importance for the future therapy in clinical medicine.

4.9 Summary

Obesity and vascular injury result in elevated leptin release in blood and the vessel wall, which can serve as a chemokine for Sca-1$^+$ progenitor cell migration. During this process, the binding of leptin to its receptor (OBR) leads to the activation of signal pathways, e.g. STAT3-Rac1/Cdc42-ERK-FAK,

which are abrogated by OBR deficiency in animal models. The migratory response of Sca-1^{+} progenitor cells to increased leptin levels may be largely responsible for enhanced neointimal formation in injured vessels. These novel findings enhance our understanding of the mechanisms of obesity-related vascular diseases. The protective role of Lepr$^{-/-}$ Sca-1^{+} progenitors also suggests a possibility to specifically target the leptin receptor in the Sca-1^{+} progenitor cells to prevent vascular remodeling.

Chapter 5
Publications

5.1 Articles

5.1.1 Published

1) **Xie Y**, Fan Y, and Xu Q. Vascular regeneration by stem/progenitor cells. *Arterioscl. Thromb. Vasc. Biol.* 2016:*36*:e33-e40 (Review).

2) Gu W, **Xie Y**, and Xu Q. Animal Models of to study pathophysiology of the vasculature. In The ESC Textbook of Vascular Biology. Krams R and Back M (Eds). Oxford University Press. p53-68, 2017 (Book Chapter).

3) Kokkinopoulos I, Wong MM, Potter CMF, **Xie Y**, Yu B, Nowak W, Le Bras A, Ni Z, Zhou C, Karamariti E, Hu Y, Zhang L, Xu Q. Single cell sequencing of vascular Sca-1^{+} progenitors revealed novel mechanisms of cell migration in response to hyperlipidaemia. *Stem Cell Reports.* In press, 2017 (Original Article).

5.1.2 In Press

4) **Xie Y**, Potter C, Le Bras A, Nowak W, Gu W, Issa Bhaloo S, Zhang Z, Hu Y, Zhang L, Xu Q. Leptin Induces Sca-1^{+} Progenitor Cell Migration Enhancing Neointimal Lesions in Vessel-Injury Mouse Models. Submitted (Accepted), 2017 (Original Article).

5.1.3 Submitted or In Preparation

5) Gu W, Nowak W, **Xie Y**, Hu Y, Zhang L, Xu Q. Perivascular adipose tissue derived stem cells are able to differentiate towards smooth muscle cells and endothelial cells and contribute to vasa vasorum formation and vascular repair. In Preparation.

6) **Xie Y**, Nowak W, Gu W, Hu Y, and Xu Q. Adipose tissue influences vascular remodeling of injured arteries *via* adipokine release and cell

migration. In preparation.

5.2 Meeting Abstracts

1) **Xie Y**, Potter C, Le Bras A, Hu Y, Zhang L, and Xu Q. Leptin Induces Sca-1$^+$ Progenitor Cell Migration Enhancing Neointimal Lesions in Vessel-Injury Mouse Models. The SmArteR 2016 Symposium, Nov 7-9, 2016, Fribourg, Switzerland.

2) **Xie Y**, Potter C, Le Bras A, Nowak W, Gu W, Hu Y, Zhang L, Xu Q. Leptin Induces Sca-1$^+$ Progenitor Cell Migration Enhancing Neointimal Lesions in Vessel-Injury Mouse Models. The Annual Conference of the British Cardiovascular Society, Jun 5-7, 2017, Manchester, UK.

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